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BRITISH PHARMACOLOGICAL SOCIETY

WINTER MEETING, 9th to 10th JANUARY, 1959, AT THE NATIONAL INSTITUTE FOR MEDICAL RESEARCH, MILL HILL, LONDON

COMMUNICATIONS

1. **A. Spinks** (*Imperial Chemical Industries Limited, Pharmaceuticals Division, Macclesfield, Cheshire*).

Effects of drugs on the twitch response of mice to solitary confinement.

2. **R. B. Barlow, I. Khan** and **R. P. Stephenson** (*Pharmacological Laboratory, University of Edinburgh*).

Tryptamine and 5-hydroxytryptamine receptors in the rat fundus strip.

3. **F. M. Sullivan** and **J. M. Robson** (*Department of Pharmacology, Guy's Hospital Medical School, London, S.E.*1).

Interrelation between glucocorticoids and isoniazid in experimental tuberculosis.

4. **E. Poulson, A. C. E. Wander** and **J. M. Robson** (*Department of Pharmacology, Guy's Hospital Medical School, London, S.E.*1).

Antagonism of oestrogen induced growth by 6-diazo-5-oxo-norleucine (DON).

5. **D. R. Laurence** and **R. A. Webster** (*Pharmacological Laboratory, University College, London, W.C.*1).

Phenothiazine tachyphylaxis in tetanus.

6. **M. W. Smith** and **M. Ginsburg** (*Department of Pharmacology, University of Bristol*).

The fate of oxytocin and oxytocin analogues.

7. **F. Hobbiger** (*Department of Pharmacology, Middlesex Hospital Medical School, London, W.*1).

New oximes for the treatment and prevention of organophosphate poisoning.

8. **F. Stothers, F. McL. Carey** and **J. J. Lewis** (*Department of Materia Medica and Therapeutics, The University, Glasgow*).

The effects of variation of the interquaternary distances and alkyl group size on some quaternary ammonium derivatives with muscle relaxant properties.

9. **M. Horlington** and **Mary F. Lockett** (*Department of Physiology and Pharmacology, Chelsea College of Science and Technology, London, S.W.*3).

Antagonism by morphine and pethidine of the analgesic actions of normorphine and norpethidine when these drugs are injected intracisternally into mice.

10. **P. A. Nasmyth** and **W. H. H. Andrews** (*Department of Pharmacology, St. Mary's Hospital Medical School, Paddington, London, W.*2).

Block by cocaine of the effect of choline 2 : 6 xylyl ether bromide (T.M.10) on the sympathetic nervous system.

11. **E. Muscholl** (*introduced by* **Marthe Vogt**) (*Department of Pharmacology, Mainz*).

The effect of drugs on the catecholamines of the heart.

12. **W. A. M. Duncan** and **J. Raventós** (*Imperial Chemical Industries Limited, Pharmaceuticals Division, Macclesfield, Cheshire*).

Pharmacodynamics of halothane.

13. **H. W. Kosterlitz** and **D. W. Taylor** (*Physiology Department, University of Aberdeen*).

The effect of morphine on vagal inhibition of the heart.

14. **Marta Weinstock, H. C. Stewart** and **K. R. Butterworth** (*Department of Pharmacology, St. Mary's Hospital Medical School, Paddington, London, W.*2).

Some observations on the mechanism of drug-induced lenticular opacities.

15. **G. F. Somers** (*The Distillers Company* (*Biochemicals*) *Limited, Speke, Liverpool*, 24).

The pharmacological properties of thalidomide.

16. **D. M. Brown** and **J. G. Bainbridge** (*introduced by* **C. A. Keele**) (*Beecham Research Laboratories Limited, Betchworth, Surrey*).

Ganglionic blocking activity of anti-acetylcholine drugs.

17. **R. S. H. Finney, A. L. Tarnoky** and **J. H. Wilkinson** (*Department of Biology, Leicester College of Technology and Commerce*).

The sodium salt of glycyrrhetinic acid dihydrogen succinate.

18. **G. V. R. Born** and **Janet Bricknell** (*The Nuffield Institute for Medical Research, Oxford*).

Uptake of 5-hydroxytryptamine by *Taenia coli* of the guinea-pig.

19. **H. P. Rang** (*introduced by* **H. O. Schild**) (*Pharmacological Laboratory, University College, London, W.C.*1).

Unspecific drug action.

20. **A. C. Frazer, R. F. Fletcher, H. G. Sammons, B. Shaw** and **R. Schneider** (*Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham*, 15).

Studies on the enteropathy caused by wheat gluten.

21. **R. Schneider, Hilary Bishop, B. Shaw** and **A. C. Frazer** (*Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham*, 15).

Studies on the effect of wheat gluten fractions on motility in isolated intestinal preparations.

22. **D. A. Brown, B. N. C. Prichard** and **J. P. Quilliam** (*Department of Pharmacology, The Medical College of St. Bartholomew's Hospital, London, E.C.*1).

Actions of staphylococcal α-toxin on gut.

23. **E. Bülbring, G. Burnstock** and **E. Gill** (*Department of Pharmacology, Oxford*).

Membrane potential changes associated with tachyphylaxis and with potentiation of drug effects in smooth muscle.

24. **D. S. Munro** (*introduced by* **G. M. Wilson**) (*Department of Pharmacology and Therapeutics, The University, Sheffield*, 10).

Effect of various human sera on the discharge of radioiodine from the mouse thyroid.

25. **B. B. Newbould** and **R. Kilpatrick** (*introduced by* **G. M. Wilson**) (*Department of Pharmacology and Therapeutics, The University, Sheffield*, 10).

Observations on some long-acting sulphonamides.

26. **E. Letley** (*introduced by* **G. Brownlee**) (*Department of Pharmacology, University of London, King's College, Strand, London, W.C.*2).

The effects of temperature on electrical stimulation and drug action on the isolated denervated rat diaphragm.

27. **H. Remmer** (*introduced by* **G. A. H. Buttle**) (*Berlin*).

The acceleration of breakdown of drugs by microsomes under the influence of barbiturates.

28. **E. Horton** (*introduced by* **W. Feldberg**) (*National Institute for Medical Research, London, N.W.*7).

The presence in human urine of a substance relaxing the rat uterus.

29. **W. Feldberg** and **B. Kovacs** (*National Institute for Medical Research, London, N.W.*7).

Reduced histamine sensitivity of the guinea-pig ileum after intraperitoneal or subcutaneous injection of extracts prepared from the white blood cell layer.

30. **T. J. Sullivan** (*introduced by* **R. S. Stacey**) (*Department of Pharmacology and Therapeutics, St. Thomas's Hospital Medical School, London, S.E.*1).

The effects of metallic edetates on the growth and blood formation of rats.

31. **S. E. Smith** (*introduced by* **R. S. Stacey**) (*Department of Pharmacology and Therapeutics, St. Thomas's Hospital Medical School, London, S.E.*1).

The sensitivity of isolated intestine following reserpine.

32. **M. K. Paasonen** (*introduced by* **P. B. Dews**) (*Department of Pharmacology, University of Helsinki, Finland*).

Increase of 5-hydroxytryptamine in the rat brain by raunescine.

33. **R. Fielden** (*introduced by* **G. A. Mogey**) (*Department of Pharmacology, The School of Medicine, Leeds*, 2).

An unusual cat disease? (*Including two-minute film.*)

34. **J. H. Burn** and **M. J. Rand** (*Department of Pharmacology, Oxford*).

Supersensitivity of the iris and the spleen to noradrenaline after sympathetic degeneration.

35. **P. J. Goodford** and **H. R. Ing** (*Department of Pharmacology, Oxford*).

The pharmacology of the ethanesulphonate anion.

DEMONSTRATIONS

1. **D. M. Lindsay, L. Sinclair** and **J. M. Robson** (*Department of Pharmacology, Guy's Hospital Medical School, London, S.E.*1).

A technique for the instillation of drugs into the cerebral ventricles of the conscious mouse.

2. **S. M. Holland, G. Pennington, J. Roberts** and **G. F. Somers** (*The Distillers Company (Biochemicals) Limited, Speke, Liverpool*, 24).

Blood and urine levels following intramuscular administration of potassium phenoxymethylpenicillin.

3. **E. Crabbe** and **G. F. Somers** (*The Distillers Company (Biochemicals) Limited, Speke, Liverpool*, 24).

An automatic rabbit cage.

4. **S. S. Adams** and **B. V. Heathcote** (*Boots Pure Drug Company Limited, Biology Division, West Bridgford, Notts*).

The effect of various sulphated polysaccharides on bone growth in the rabbit.

5. **E. Horton** (*introduced by* **W. Feldberg**) (*National Institute for Medical Research, London, N.W.*7).

The use of the uterus and duodenum of the rat for the estimation of kinins.

6. **K. Fleischhauer** and **F. K. Ohnesorge** (*introduced by* **W. Feldberg**) (*National Institute for Medical Research, London, N.W.*7).

Central actions and selective staining of certain areas of the brain by dithizone.

7. **M. Draskoci, W. Feldberg, K. Fleischhauer** and **P. S. R. K. Haranath** (*National Institute for Medical Research, London, N.W.*7).

Absorption of histamine from the cerebral ventricles.

8. **M. Draskoci** and **P. S. R. K. Haranath** (*introduced by* **W. Feldberg**) (*National Institute for Medical Research, London, N.W.*7).

Potentiation of the acetylcholine contraction of the rat uterus by minute doses of 5-hydroxytryptamine.

9. **J. H. Burn, E. H. Leach, M. J. Rand** and **J. W. Thompson** (*Departments of Pharmacology and Physiology, University of Oxford, and the Department of Pharmacology, Royal College of Surgeons*).

Peripheral effects of nicotine and acetylcholine probably exerted on chromaffin tissue in (*a*) the rabbit ear (vasoconstriction), (*b*) the cat's nictitating membrane (contraction), (*c*) the cat's tail (pilomotor response).

10. **C. A. Joslin** (*introduced by* **J. B. E. Baker**) (*Department of Pharmacology, Charing Cross Hospital Medical School*).

A transistorized stimulator.

Brit. J. Pharmacol. (1959), **14**, 4.

JAMES ANDREW GUNN

C.B.E., M.D., D.Sc., D.M., F.R.C.P.

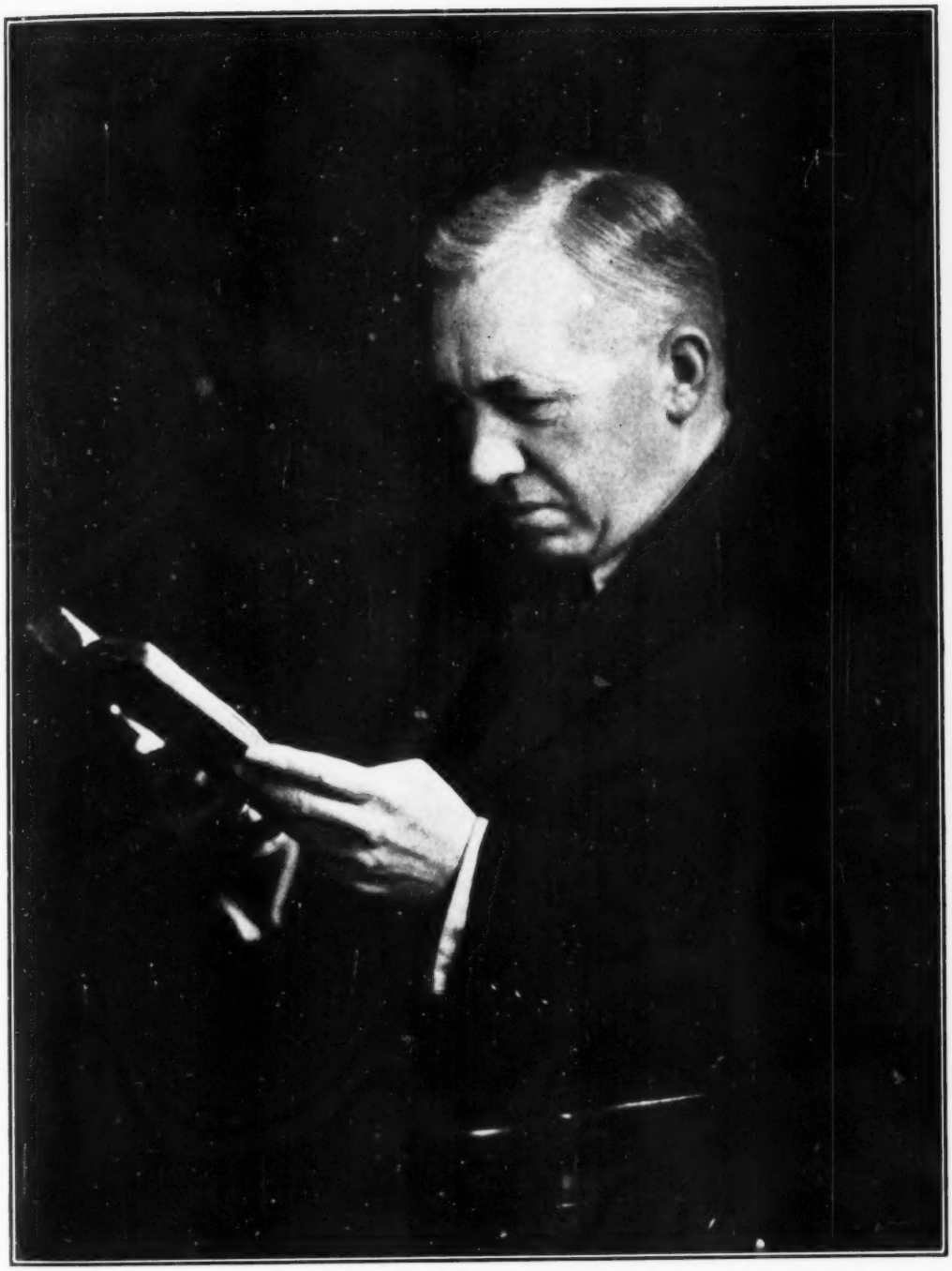
Professor J. A. Gunn was the father of the British Pharmacological Society, and his death on October 21, 1958, caused great sorrow among his many friends. He was born at Kirkwall on January 26, 1882, and never lost his attractive Orcadian accent. He attended Kirkwall School, where he learned to write good simple prose and to tell a story well. Gunn was always ready to acknowledge how much he owed to the influence of his father, a well-known ornithologist in the Orkneys. At Edinburgh University he won many prizes and distinctions as a medical student and had acquired five degrees by the time he was twenty-eight. He had some experience of medical practice in the islands of Scotland and then joined the staff of the Department of Materia Medica in Edinburgh under the famous Sir Thomas Fraser, who was by this time a busy physician. Gunn and Sillar did all the teaching, and Gunn was active in research on arsenic, yohimbine, cobra venom, and harmine. In 1912 he was awarded a Beit Fellowship and authorized to work on four different problems, but before he could do this he was appointed Reader in Pharmacology in Oxford. His lectures were popular and he started a Practical Class; and in 1917 he became Professor. He held this post for twenty years and was then Professor of Therapeutics till 1946, living and working in the building which was once the Radcliffe Observatory and is now the Nuffield Institute for Medical Research. During World War I, he served with the R.A.M.C.

Gunn's main scientific interests were the alkaloid harmine and the sympathomimetic amines. Harmine acts on plain muscle, but also on the central nervous system, where it causes hallucinations and dreams; recent speculations have suggested that these effects depend on the presence of an indole nucleus. Gunn showed that sympathomimetic amines containing a *meta*-methoxy group stimulate the central nervous system in mice and this also has become important since it has been shown that such compounds are formed in the body.

Gunn edited the 12th edition of Cushny's *Textbook of Pharmacology and Therapeutics*. His own *Introduction to Pharmacology and Therapeutics* was popular because it was short and simple, and nine editions appeared in 30 years. He was appointed Chairman of the British Pharmacopoeia Commission in November, 1939, and was responsible for six addenda to the 1932 *Pharmacopoeia* and for the 1948 *Pharmacopoeia*. This involved a great deal of hard work in difficult times, and his care and energy contributed much to the great success of this work.

In the last fifty years Pharmacology has become an important science and the British contribution to this advance owes much to Gunn. It was on his initiative that a group of about twenty pharmacologists met in Oxford in 1931 and founded the British Pharmacological Society. There seemed to be very few pharmacologists at that time, and some people thought that the subject should disappear and be merged in the Departments of Physiology and Medicine. The foundation of the Society and its vigorous growth have done much to raise the prestige, not only of Pharmacology, but also of British Medicine.

J. H. G.



James Andrew Gunn (1882-1958)

NOTICE TO CONTRIBUTORS

Manuscripts should now be submitted to Dr. J. M. Walker, Secretary to the Editorial Board, British Journal of Pharmacology, at the Department of Pharmacology, South Parks Road, Oxford.

Brit. J. Pharmacol. (1959), **14**, 5.

2-HYDROXYIMINOMETHYL-*N*-METHYLPYRIDINIUM METHANESULPHONATE AND ATROPINE IN THE TREATMENT OF SEVERE ORGANOPHOSPHATE POISONING

BY

D. R. DAVIES, A. L. GREEN AND G. L. WILLEY*

From the Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wilts

(RECEIVED JULY 31, 1958)

The soluble methanesulphonate of the oxime 2-hydroxyiminomethyl-*N*-methylpyridinium (P2S) has been used to treat animals poisoned with sarin or ethyl pyrophosphate. The effect of the size of the dose, and its time of administration in relation to poisoning, have been examined. This oxime is very efficient in conjunction with atropine when given either before or after poisoning. About 30 mg./kg. seems to be the optimum therapeutic dose of the methanesulphonate. The significance of this optimum is discussed in relation to the treatment of accidental poisoning by organophosphate insecticides in man.

In conjunction with atropine, the iodide of the oxime 2-hydroxyiminomethyl-*N*-methylpyridinium (pyridine-2-aldoxime methiodide; P2AM) is an effective antidote to organophosphate poisoning. Thus Kewitz, Wilson, and Nachmansohn (1956) showed that 50 mg./kg. of the iodide with 10 mg./kg. of atropine, given intraperitoneally, were effective against diethyl *p*-nitrophenyl phosphate (E600) or dyflos (di-*iso*propyl phosphorofluoridate). Askew (1957), using 100 mg./kg. of the iodide with 17.4 mg./kg. of atropine sulphate, treated successfully *iso*propyl methylphosphonofluoridate (sarin) poisoning, and Wilson and Sondheimer (1957) found a similar combination effective against poisoning by ethyl pyrophosphate. Further evidence of the potentially wide application of the iodide with atropine has been provided by Hobbiger (1957), by Bethe, Erdmann, Lendle, and Schmidt (1957), and by Fournel (1958).

The dose of the iodide used by all these authors was relatively large. Since the iodide is only about 4% soluble in water, the injection of high doses into man would require large volumes of solution. If given intravenously to rabbits in conjunction with atropine, it is highly effective against sarin poisoning in doses as low as 5 mg./kg. (Wills, Kunkel, Brown, and Groblewski, 1957). However, since convulsions are frequently present in severe anticholinesterase poisoning, intravenous injections even of this size will often be impossible. A more soluble salt of this oxime with a potency at least equal to that of the iodide, yet capable of being administered intramuscularly in small volumes of solution of sufficient strength, is thus highly desirable. Such a salt is the methanesulphonate (P2S), 1 g. of which is soluble in 2 to 3 ml. of water (Davies and Willey, 1958). In this paper, the therapeutic and prophylactic potentialities of this methanesulphonate have been assessed in the treatment of poisoning by sarin or ethyl pyrophosphate in laboratory animals.

METHODS

The methanesulphonate was prepared by boiling 2-hydroxyiminomethylpyridine with methyl methanesulphonate in benzene; it had an m.p. of 155°. For injection it was dissolved in water, *p*H being adjusted to 7.4 with 0.1 N-NaOH immediately before use. Atropine sulphate (B.D.H.) was always injected in the same solution as the oxime. The therapeutic agents were given intramuscularly in volumes of 3 ml./kg. body weight to mice, and 1 ml./kg. to rats, guinea-pigs and rabbits. Sarin or ethyl pyrophosphate was given subcutaneously in saline in volumes of 10 ml./kg. to mice, 1 ml./kg. to rats and guinea-pigs, and 0.5 ml./kg. to rabbits. Female mice (18 to 22 g.), rats (180 to 200 g.), guinea-pigs (350 to 400 g.) and rabbits (2 to 3 kg.) were used.

In general, the potency of any treatment was measured in terms of LD50 values, although in some

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prophylactic experiments it was more convenient to give a standard number of animals a fixed dose of the organophosphate at various intervals after the therapeutic agents.

Each LD50 was calculated by the method of moving averages (Thompson, 1947), using the tables compiled by Weil (1952). A four-point assay was usually performed, with six animals at each dose of organophosphate.

Results

The Probit Regression Line in the Presence and Absence of Treatment.—Assessments of the potency of treatment were made by comparing the LD50 of the organophosphate in the presence and absence of treatment. This is a purely arbitrary basis of comparison. It cannot be extended to the whole dose/response curves since these were not always parallel, their slopes almost always decreasing as the treatment became increasingly effective. A few examples to illustrate this decrease are given in Table I.

Table I

THE PROBIT/LOG DOSE REGRESSION LINES IN SARIN AND ETHYL PYROPHOSPHATE POISONING IN THE PRESENCE AND ABSENCE OF TREATMENT WITH 2-HYDROXYIMINOMETHYL-*N*-METHYLPYRIDINIUM METHANESULPHONATE AND ATROPINE

Treatment with oxime and atropine was given 1 min. after sarin in mice, 10 min. before sarin in rabbits, 10 min. before ethyl pyrophosphate. The values for the LD50 are given with the 95% limits.

Species	Toxic Agent	Dose of Oxime (mg./kg.)	No. Animals	LD50 (mg./kg.)	Regression Coefficient ± s.d.
Mouse	Sarin	0	40	0·305 (0·27–0·37)	10·2±2·7
		30	40	1·0 (0·79–1·30)	4·8±1·1
Rabbit	,,	0	40	0·05 (0·044–0·055)	10·1±1·9
		30	50	6·53 (4·80–9·50)	3·3±0·9
Rat ..	Ethyl pyrophosphate	0	40	0·38 (0·34–0·43)	11±2·6
		120	40	5·18 (3·60–10·9)	4·2±1·6

Insufficient animals were used in most of the LD50 determinations to show that this decrease was always statistically significant; but the trend was invariably found.

A Comparison of the Methanesulphonate and Iodide of the Oxime as Adjuvants to Atropine.—As the functional part of both salts is the 2-hydroxyiminomethyl-*N*-methylpyridinium ion, the therapeutic effectiveness of these two salts would therefore not be expected to differ significantly. That this is so against sarin poisoning is confirmed by the results given in Table II.

Table II

COMPARISON OF THE METHANESULPHONATE AND IODIDE OF 2-HYDROXYIMINOMETHYL-*N*-METHYLPYRIDINIUM AS ADJUVANTS TO ATROPINE IN SARIN POISONING

Oxime (30 mg./kg.) with atropine sulphate (17·4 mg./kg.) was given 10 min. before sarin. The values for the LD50 for sarin are given with 95% limits.

Species	Sarin LD50 (mg./kg.)		
	No Treatment	Methanesulphonate + Atropine	Iodide + Atropine
Mouse ..	0·30 (0·23–0·42)	0·98 (0·79–1·20)	1·34 (1·05–1·72)
Rat ..	0·14 (0·09–0·18)	0·45 (0·28–0·71)	0·38 (0·27–0·54)
Guinea-pig	0·048 (0·041–0·057)	3·81 (2.66–5·48)	4·65 (3·16–6·87)
Rabbit ..	0·050 (0·046–0·055)	6·12 (4·41–8·49)	6·54 (5·00–8·60)

The Therapeutic Efficacy of the Methanesulphonate with Atropine.—In these experiments "therapy" refers to treatment after poisoning and "prophylaxis" to treatment before poisoning. Table III shows the effect of 30 mg./kg. of the methanesulphonate combined with 17.4 mg./kg. of atropine sulphate, given 1 min. after sarin or ethyl pyrophosphate, in mice, rats, guinea-pigs and rabbits.

Table III

THE "THERAPEUTIC" ACTIVITY OF 2-HYDROXYIMINOMETHYL-*N*-METHYLPYRIDINIUM METHANESULPHONATE (30 MG./KG.) WITH ATROPINE SULPHATE (17·4 MG./KG.) AGAINST SARIN AND ETHYL PYROPHOSPHATE

Treatment was given intramuscularly 1 min. after the subcutaneous injection of toxic agent. The LD50 values are given with the 95% limits in mg./kg.

Species	LD50 of Toxic Agent Alone		LD50 of Toxic Agent in Treated Animals	
	Sarin	Ethyl Pyrophosphate	Sarin	Ethyl Pyrophosphate
Mouse ..	0·30 (0·22–0·42)	0·58 (0·52–0·64)	1·2 (0·73–2·2)	11·7 (9·1–15·2)
Rat ..	0·14 (0·09–0·18)	0·38 (0·34–0·43)	0·22 (0·16–0·32)	2·2 (1·6–3·2)
Guinea-pig	0·048 (0·041–0·057)	0·70 (0·37–1·3)	1·8 (1·3–2·5)	14·0 (8·7–23)
Rabbit ..	0·050 (0·046–0·055)	0·30 (0·27–0·32)	2·0 (1·6–2·5)	6·3 (5·1–7·9)

This treatment was very effective against ethyl pyrophosphate in each species and against sarin in rabbits and guinea-pigs. Rats and mice poisoned with sarin were less responsive to treatment. The effect of varying the dose of methanesulphonate, while keeping that of atropine constant, is shown in Table IV. In mice the increase in therapeutic effect with increasing dose of methanesulphonate fell off rapidly once the dose exceeded 30 mg./kg. This tendency was less marked in rats.

TABLE IV

THE EFFECT OF DOSE UPON THE "THERAPEUTIC" POTENCY OF 2-HYDROXYIMINOMETHYL-*N*-METHYLPYRIDINIUM METHANESULPHONATE IN THE PRESENCE OF A CONSTANT DOSE OF ATROPINE

The dose of atropine sulphate was 17·4 mg./kg. The LD50 values are given with 95% limits in mg./kg. 0 indicates that neither oxime nor atropine was given.

Toxic Agent	Methanesulphonate (mg./kg.)	LD50	
		Rats	Mice
Sarin ..	0	0·14 (0·09–0·18)	0·30 (0·22–0·42)
	15	—	0·93 (0·58–1·5)
	30	0·22 (0·16–0·32)	1·20 (0·73–2·2)
	120	0·59 (0·34–1·0)	1·13 (0·79–1·6)
Ethyl pyrophosphate	0	0·38 (0·34–0·43)	0·58 (0·52–0·64)
	10	1·8 (1·1–2·9)	2·8 (1·7–4·6)
	30	2·2 (1·6–3·2)	11·7 (9·1–15·2)
	120	5·0 (3·6–7·1)	11·3 (7·1–18·0)

The Prophylactic Efficacy of the Methanesulphonate with Atropine

The protective effect of the methanesulphonate (30 mg./kg.) with atropine sulphate (17.4 mg./kg.) given 10 min. before poisoning is shown in Table V. In general, treatment before poisoning was more effective than treatment after poisoning. The LD50 of ethyl pyrophosphate was raised 20- to 50-fold in each species; the LD50 of sarin was increased 70 to 100 times in rabbits and guinea-pigs, but only 3 to 4 times in rats and mice. In contrast to the "therapeutic" experiments, the prophylactic effect of the methanesulphonate increased approximately linearly with increasing dose in both rats and mice (see Table VI).

The Influence of the Interval Between Treatment and Poisoning

The two purposes of these experiments were, first, to determine how long the protective action of a given dose of the methanesulphonate lasted, and, second, to see whether there was an optimum time between treatment and poisoning. The maximum dose of the methanesulphonate that could be given without observable side-effects was 120 mg./kg. (Davies and Willey, 1958). At this dose some protection was still obtained in rats, even when the organophosphate was given more than 3 hr. after treatment; in mice, however, protection had almost disappeared after less than 2 hr. (Table VII). The duration of the protective effect fell off fairly rapidly as the dose of the methanesulphonate was decreased. The optimum interval between treatment and poisoning was greater at the highest dose of the methanesulphonate, but a 10 min. interval almost always gave nearly optimal results. Very similar results were obtained in a few experiments with ethyl pyrophosphate.

TABLE V

THE "PROPHYLACTIC" ACTIVITY OF 2-HYDROXYIMINOMETHYL-*N*-METHYLPYRIDINIUM METHANESULPHONATE (30 MG./KG.) WITH ATROPINE SULPHATE (17·4 MG./KG.) AGAINST SARIN AND ETHYL PYROPHOSPHATE

Treatment was given intramuscularly 10 min. before the subcutaneous injection of the toxic agent. The LD50 values with 95% limits are given in mg./kg.

Species	LD50 of Toxic Agent Alone		LD50 of Toxic Agent in Treated Animal	
	Sarin	Ethyl Pyrophosphate	Sarin	Ethyl Pyrophosphate
Mouse ..	0·30 (0·22–0·42)	0·58 (0·52–0·64)	1·1 (0·79–1·9)	20 (16–25)
Rat ..	0·14 (0·09–0·18)	0·38 (0·34–0·43)	0·45 (0·28–0·71)	17 (7·8–30)
Guinea-pig	0·048 (0·041–0·050)	0·70 (0·4–1·3)	3·5 (2·3–5·5)	35 (20–60)
Rabbit ..	0·050 (0·046–0·055)	0·30 (0·27–0·32)	5·1 (3·0–8·5)	8·4 (6·0–11·6)

TABLE VI

THE EFFECT OF DOSE UPON THE "PROPHYLACTIC" POTENCY OF 2-HYDROXYIMINOMETHYL-*N*-METHYLPYRIDINIUM METHANESULPHONATE IN THE PRESENCE OF A CONSTANT DOSE OF ATROPINE

The dose of atropine sulphate was 17·4 mg./kg. The LD50 values with 95% limits are given in mg./kg. 0 inicates that neither oxime nor atropine was given.

Toxic Agent	Oxime mg./kg.	LD50	
		Rats	Mice
Sarin	0	0·14 (0·09–0·18)	0·30 (0·22–0·42)
	30	0·45 (0·28–0·71)	1·1 (0·79–1·9)
	120	1·4 (1·1–1·9)	5·0 (3 8–6·6)
Ethyl pyrophosphate	0	0·38 (0·34–0.43)	0·58 (0·52–0·64)
	10	2·5 (2·0–3·2)	5·3 (4·0–7·0)
	30	17 (7·8–30)	20 (16–25)
	90	—	37 (26–64)
	120	36 (22–57)	95 (66–138)

TABLE VII

EFFECT OF TIME BETWEEN ADMINISTRATION OF VARIED DOSES OF 2-HYDROXYIMINOMETHYL-*N*-METHYLPYRIDINIUM METHANESULPHONATE AND ATROPINE AND INJECTION OF SARIN

Atropine sulphate, 17·4 mg./kg., was given. Dose of sarin in rats, 0·75 mg./kg. (5·4 × LD50); and in mice, 1·2 mg./kg. (4.0 × LD50). Numerals in table give mortalities in groups of 25 animals.

Methanesulphonate mg./kg.	Time (min.)						
	200	100	50	10	5	−1	−5
Rats							
120	19	6	3	11	11	15	25
60	—	21	15	16	12	23	—
30	—	23	15	18	15	23	—
15	—	—	23	20	19	24	—
Mice							
120	—	23	1	4	6	12	25
60	—	25	22	5	10	8	—
30	—	—	23	17	13	17	—
15	—	—	23	14	14	19	—

DISCUSSION

In this paper the efficacy of the methanesulphonate of 2-hydroxyiminomethyl-*N*-methylpyridinium with atropine in the therapeutic and

prophylactic treatment of animals poisoned with sarin and ethyl pyrophosphate has been reported. When the methanesulphonate (30 mg./kg.) and atropine were given therapeutically, the LD50 of ethyl pyrophosphate was raised 20-fold in mice, guinea-pigs and rabbits, although only 5-fold in rats. The LD50 of sarin was raised 40-fold in guinea-pigs and rabbits, but only about 4-fold in mice, and still less in rats. Except in rats poisoned with sarin, increasing the dose above 30 mg./kg. produced no proportionate increase in effect. Prophylactically this limiting effect of increased dose was not apparent: the consequence of increasing the dose was particularly striking on the duration of the prophylactic effect (see Table VII). A similar species variation was noticed as in the therapeutic experiments, especially in the resistance to treatment of rats and mice poisoned with sarin. This anomaly in the behaviour of rats was particularly striking in the light of earlier therapeutic experiments with the oximes hydroxyiminoacetone and diacetyl monoxime (Askew, 1956, 1957). These two compounds, especially the latter, were far more effective against sarin poisoning in rats than in any other species.

The apparent optimal therapeutic dose of 30 mg./kg. may be related to the fact that reactivation by the methanesulphonate reached a maximum rate at quite low concentrations (Green and Smith, 1958). Prophylactically, the higher doses might enable a fairly persistent optimal concentration to be maintained in the body tissues.

The value of these results depends upon the extent to which they can be applied to man, and here factors other than the intrinsic potency of the oxime and atropine are important. For example, the very high water solubility of the methanesulphonate gives it a distinct advantage over the iodide in ease of administration. In accidental insecticide poisoning, therapy will be more important than prophylaxis, and in these circumstances the possible existence of a relatively low optimal single dose (30 mg./kg.) of the methanesulphonate is decidedly helpful. The administration of a dose of this magnitude intramuscularly to human beings, about 2 g./man, would probably be a practical field procedure.

Namba and Hiraki (1958) have, in fact, successfully given 1 g. of the iodide (in 20 ml. water) intravenously to agricultural workers accidentally poisoned with parathion; but in very severe poisoning accompanied by convulsions, though intravenous injections would be impossible, intramuscular injections would still be practicable.

As a prophylactic measure, the regular injection of the methanesulphonate is unlikely to be widely applicable. In rats and mice even large doses, of more than 100 mg./kg., only provide substantial protection for about 2 hr. However, recent experiments seem to show that a satisfactory form of "prophylactic" treatment in rats is to give the oxime orally and prophylactically, and the atropine intramuscularly and therapeutically (Creasey, personal communication).

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PLASMA ADRENALINE AND NORADRENALINE AFTER PHENOXYBENZAMINE ADMINISTRATION, AND DURING HAEMORRHAGIC HYPOTENSION, IN NORMAL AND ADRENALECTOMIZED DOGS

BY

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The intravenous administration of the antiadrenaline drug phenoxybenzamine (Dibenzyline) markedly raised the arterial adrenaline and noradrenaline concentration in dogs lightly anaesthetized with thiopentone. Graded haemorrhage led to a further rise in the amounts of amine. In adrenalectomized dogs, phenoxybenzamine moderately increased the plasma noradrenaline concentration. During haemorrhagic hypotension, previous treatment of adrenalectomized animals with phenoxybenzamine led to a significantly greater rise in plasma noradrenaline compared with that of adrenalectomized animals subjected to haemorrhage without treatment with phenoxybenzamine. Thus, phenoxybenzamine (1) raised plasma amine concentration largely due to adrenal medullary stimulation, and (2) led to increased plasma noradrenaline concentrations during sympathetic stimulation in adrenalectomized animals. The previous administration of phenoxybenzamine reduced the amount of blood which could be withdrawn before final circulatory collapse in both normal and adrenalectomized dogs.

Brown and Gillespie (1957) have recently reported that the administration of phenoxybenzamine increased the concentration of noradrenaline in splenic venous blood following splenic nerve stimulation in cats, and they have suggested that inactivation of sympathetic receptor sites by phenoxybenzamine allowed an accumulation and overflow of transmitter substance. In addition it has been shown by Benfey, Ledoux, and Melville (1958) that the administration of phenoxybenzamine to dogs greatly augmented the urinary excretion of noradrenaline and adrenaline.

This study reports arterial plasma adrenaline and noradrenaline concentrations before and after phenoxybenzamine administration in normal and adrenalectomized dogs. The effect of graded haemorrhagic hypotension induced after phenoxybenzamine injection has also been investigated. In addition, observations have been made during haemorrhagic hypotension induced in adrenalectomized dogs not previously treated with phenoxybenzamine.

METHODS

Eight dogs were very lightly anaesthetized with minimal amounts of thiopentone. A femoral artery was cannulated following infiltration of the groin with 5 to 10 cc. of 1% procaine hydrochloride. Heparin (5 mg./kg.) was then given intravenously. Arterial blood pressure was recorded continuously by means of a Statham strain gauge and Sanborn recorder. Arterial blood samples were withdrawn immediately before and after periods of 1 hr. and 2 hr. following the intravenous injection of phenoxybenzamine (20 mg./kg. given over 5 min.). Thereafter, a state of progressive haemorrhagic hypotension was induced by withdrawal of 12 ml./kg. of blood every 15 min. until circulatory collapse occurred.

Five healthy dogs were subjected to bilateral lumbar adrenalectomy, the operations being done in two stages at intervals of one week. Daily intramuscular injections of cortisone (0.5 mg./kg.) and desoxycorticosterone acetate (0.1 mg./kg.) were maintained for the five to seven days preceding each study using phenoxybenzamine which was performed in a similar manner to that described for the dogs with intact adrenal glands, except that more blood samples were taken for assay before induction of haemorrhagic hypotension.

Three adrenalectomized dogs were also subjected to graded haemorrhage as described previously (Millar and Benfey, 1958) by withdrawal of 12 ml./kg. of blood at intervals of 1 hr. until 36 ml./kg. had been lost, following which blood was withdrawn

at intervals of 15 min. until circulatory failure developed.

Samples withdrawn in the period before and after phenoxybenzamine administration (before haemorrhage) were limited to 30 to 40 ml., yielding the 15 to 20 ml. of plasma required for a single determination of adrenaline and noradrenaline concentration; the estimated values obtained from these samples therefore represent single assays. Subsequent samples (12 ml./kg.) from the normal and adrenalectomized dogs injected with phenoxybenzamine and the adrenalectomized dogs subjected to haemorrhage without phenoxybenzamine pretreatment were estimated in duplicate. Blood samples were withdrawn into glass tubes containing heparin and centrifuged for 15 to 20 min.

The fluorimetric method employed for estimation of adrenaline and noradrenaline has been outlined elsewhere (Millar and Benfey, 1958), but certain modifications were made for these studies.

Chromatographic Adsorption of Catechol Amines on Alumina

This was conveniently performed with a glass column consisting of a bulbous middle portion (capacity 30 to 50 ml.) with upper and lower limbs, the lower limb being narrower and constricted at its lower end. After placing a glass wool plug at the constriction in the lower limb, the column was partly filled with distilled water into which was poured 500 mg. of alumina (acid-washed, Merck). This was carefully stirred with a thin glass rod to eliminate air bubbles. The level of water was lowered to just above the alumina by applying air pressure to the upper limb of the column; 20 ml. of glass-distilled water was then run through to the same level. This was followed by the plasma sample (15 to 20 ml. from blood containing heparin) which was run through undiluted, or diluted with an equal volume of glass-distilled water; the plasma may be previously adjusted to *p*H 8.4 by gentle stirring and the addition of a few drops of 1% NaOH. It was run through the column at a rate of about 30 drops/min. If clogging occurred the upper surface of the alumina was stirred gently with a glass rod.

When the plasma level had almost reached the upper part of the alumina, 10 ml. of glass-distilled water was added and washed through, care being taken to avoid drying of the column. The adsorbed amines were eluted with 4 ml. of acetic acid (0.1N); recoveries 10 to 15% higher were obtainable by eluting with a second 4 ml. of acid.

The first and second 4 ml. eluates were collected in separate 15 ml. glass-stoppered tubes, and each eluate divided into 2 ml. portions for "sample" and "blank" fractions.

Recoveries, which should be above 70%, depended greatly on the technique of chromatographic adsorption, which must be studied carefully with the particular brand of alumina employed, and even with each individual batch. Although acid-washed alumina reduced the *p*H of the plasma percolate, so that satisfactory adsorption of catechol amines must occur to some extent at a *p*H lower than 8, so far this variety has given better results than the almost neutral type, which showed higher blank values unless specially prepared. The use of more concentrated acetic acid for elution from the column may be preferred, but the *p*H of the eluate/sodium acetate buffer mixture (see below) must then be brought to *p*H 6 to ensure complete oxidation of noradrenaline on addition of potassium ferricyanide.

Conversion of Adrenaline and Noradrenaline to Fluorescent Trihydroxyindole Compounds

The procedure resembled that of Lund (1950).

Treatment of "Sample" Fraction.—Reagents were added in the following sequence: (a) 1.0 ml. of 3 M-sodium acetate buffer (*p*H 8.0); (b) 0.1 ml. of 0.1% potassium ferricyanide and left for 2 min.; (c) 0.5 ml. of NaOH (20%), containing 1/10 of 2% ascorbic acid.

Treatment of "Blank" Fraction.—(a) 1.0 ml. of sodium acetate buffer; (b) 0.5 ml. of NaOH and ascorbic acid; (c) 0.1 ml. of 0.1% potassium ferricyanide.

Adrenaline and noradrenaline standard solutions, containing 0.05 μg. base/2 ml. acetic acid (0.1N), were run through the estimation procedure together with standard "blanks" as described. With the acid-washed alumina employed for these studies, it has not been found necessary to use acetic acid eluates from alumina columns for adrenaline and noradrenaline standard solutions.

Differential Estimation of Adrenaline and Noradrenaline

Fluorescence was measured 3 to 5 min. after addition of NaOH to the "sample" fraction. A Farrand or other photofluorimeter of comparably high sensitivity with the following filter arrangements allowed differential estimation of adrenaline and noradrenaline: (1) Primary filter at 400 mμ. with Wratten filter no. 35; secondary filter at 500 mμ. with Wratten no. 57. (2) Primary filter at 436 mμ. with Wratten no. 35; secondary filter at 500 mμ. with Wratten no. 57.

Two equations are constructed, where *a*, *b*, *c*, *d* represent the number of galvanometer divisions/0.01 μg. of adrenaline (A) and noradrenaline (N) for the two filter arrangements. "Net fluorescence" is the fluorescence of the sample minus that of the blank.

1. $aA + bN$ = Net fluorescence at 400 mμ. primary wavelength.

2. $cA + dN$ = Net fluorescence at 436 mμ. primary wavelength.

Solution of these equations gives the adrenaline and noradrenaline concentrations in μg./l. of plasma, when 20 ml. of plasma was used for assay and the eluates divided into two equal fractions (the "net fluorescence" values for each eluate were added). Smaller or larger plasma samples require the use

TABLE I

PLASMA ADRENALINE AND NORADRENALINE CONCENTRATIONS BEFORE AND AFTER PHENOXYBENZAMINE AND DURING HAEMORRHAGIC HYPOTENSION INDUCED AFTER THE 120 MIN. SAMPLE

The concentrations of adrenaline (A) and noradrenaline (N) are expressed in μg./l. Paired values in lowest line of the Table represent mean arterial blood pressure (B.P.) before and after withdrawal of each sample at each time.

Expt. No.	Before		Time (in Minutes) after Injection of 20 mg./ml. of Phenoxybenzamine											
			60		120		135		150		165		180	
	A	N	A	N	A	N	A	N	A	N	A	N	A	N
1	0·15	0·42	6·5	3·6	7·7	9·1	7·4	8·5	11	6·2				
2	0·18	0·04	1·7	1·6	1·2	4·5	2·9	4·3	3·3	3·9	4·9	4·8	19	6·4
3	0·00	0·00	6·6	2·7	4·9	5·2	9·8	7·3	9·2	7·4	20	9·9		
4	0·18	0·37	2·3	0·36	3·8	0·68	5·4	0·76	13	2·0	27	6·6		
5	0·10	0·20	0·71	0·27	1·9	0·47	3·2	0·65	6·2	1·1	14	2·7		
6	0·00	0·00	0·59	0·29	2·6	0·97	13	3·0	2·2	1·2				
7	0·12	0·09	1·8	1·5	2·6	1·7	3·1	1·3	4·0	1·3	9·4	2·7	27	11
8	0·05	0·29	10·0	2·0	9·3	2·1	12	5·5	9·2	6·2				
Mean ..	0·10	0·18	3·8	1·5	4·3	3·1	7·1	3·9	7·3	3·7	15	5·3	23	8·7
B.P. ..	143	146	95	80	70	65	63	35	42	22	26	14	17	

of a correction factor. Similar techniques have recently been described by Price and Price (1957) and Cohen and Goldenberg (1957).

This method allowed the estimation of less than 0.002 μg. adrenaline or noradrenaline, with an accuracy in mixtures of the order of ±25%. The physiological meaning of estimated plasma levels of adrenaline and noradrenaline which are close to zero is at present unknown, but serial quantitative changes, which are statistically significant, can be measured at levels below 0.5 μg./l.

RESULTS

In eight normal dogs the injection of phenoxybenzamine (20 mg./kg.) was followed by marked increases in arterial adrenaline and noradrenaline concentrations. Table I gives the results from individual experiments; the mean values are plotted in Fig. 1. From a mean adrenaline concentration of 0.10 μg./l. in the control samples there was an increase to 3.8 at 60 min. and 4.3 at 120 min. Noradrenaline increased from a mean value of 0.18 μg./l. before phenoxybenzamine injection to 1.5 and 3.1 after 60 and 120 min. respectively.

When the dogs were subjected to graded haemorrhagic hypotension by withdrawal of blood at 15 min. intervals starting 135 min. after phenoxybenzamine administration, the mean adrenaline concentration of the last samples obtained in each experiment increased to 16 μg./l. The increase in noradrenaline concentration was less pronounced, the final mean value being 6.2 μg./l.

Table II shows that, after phenoxybenzamine injection in five adrenalectomized dogs, there was no significant increase in plasma adrenaline concentration. The noradrenaline concentration, however, increased moderately in three out of five dogs after phenoxybenzamine had been given. More marked increases in plasma noradrenaline occurred when severe haemorrhagic hypotension was induced by withdrawal of blood at 15 min. intervals. The mean noradrenaline level in the final samples withdrawn before complete circulatory collapse was 2.4 μg./l., which differed significantly from the mean noradrenaline concentration of 0.19 μg./l. before injection of phenoxybenzamine (Fisher-Behrens test, $P<0.01$). By contrast the adrenaline plasma concentrations were not consistently altered in haemorrhagic hypotension, as is also shown in Table II.

In the three adrenalectomized dogs subjected to graded haemorrhage without prior injection

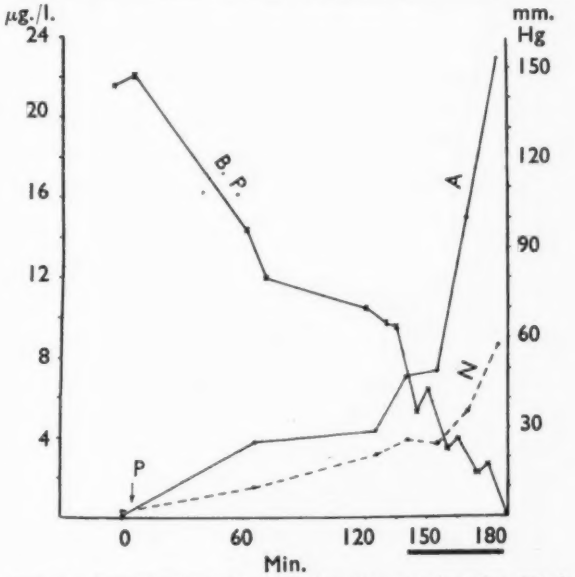

FIG. 1.—Concentrations of adrenaline (A), noradrenaline (N) in arterial blood plasma and mean arterial blood pressure (B.P.) (diastolic blood pressure +1/3 of pulse pressure) before and after phenoxybenzamine (20 mg./kg. being injected at P) and graded haemorrhage (horizontal bar).

TABLE II

PLASMA ADRENALINE AND NORADRENALINE CONCENTRATION IN ADRENALECTOMIZED DOGS BEFORE AND AFTER PHENOXYBENZAMINE, AND DURING HAEMORRHAGIC HYPOTENSION

The concentrations of adrenaline (A) and noradrenaline (N) are expressed in μg./l. Paired values in lowest line of each group represent mean arterial blood pressure (B.P.) before and after withdrawal of each blood sample. Asterisks indicate 12 mg./kg. of blood was withdrawn.

Expt. No.	Before	Time (in Minutes) after Phenoxybenzamine (10 mg./kg.)					
		60	120	180	195	210	225
	A N	A N	A N	A N	A N	A N	A N
1	0·13 0·10	0·03 0·43	0·07 0·55	0·17 0·55		0·00 1·3	0·14* 1·2*
2	0·12 0·06	0·07 0·24	0·07 0·32	0·11 0·47	0·09 0·59	0·10* 1·1*	0·12* 2·6*
3	0·27 0·12	0·38 0·61	0·41 0·94	0·12 0·77	0·11* 1·2*	0·27* 1·5*	0·22* 1·9*
Mean ..	0·17 0·09	0·16 0·43	0·18 0·60	0·13 0·60			0·16* 1·9*
B.P. ..	74 72	65 62	45 49	54 50			27
Expt. o.	Before	Time (in Minutes) after Phenoxybenzamine (20 mg./kg.)					
		60	120	135	150	165	
	A N	A N	A N	A N	A N	A N	
4	0·38 0·37	0·35 0·26	0·47 0·28	0·38* 0·71*	0·51* 2·1*		
5	0·04 0·32	0·04 0·32	0·20 0·29	0·11* 0·49*	0·30* 1·1*	0·81* 4·1*	
Mean ..	0·21 0·35	0·20 0·29	0·34 0·29	0·25* 0·60*	0·42* 1·6*		
B.P. ..	119 116	104 77	74 51	71 27	41 13	32	

of phenoxybenzamine the plasma noradrenaline concentration increased progressively, as shown in Table III. The noradrenaline content of the final samples, withdrawn just before circulatory collapse, was in each case significantly greater than that of the first sample, as shown by analysis of variance using duplicate paired samples. The mean noradrenaline concentration in the final samples of this series (0.57 μg./l.) was significantly different ($P<0.05$) from the final mean noradrenaline concentration (2.4 μg./l.) in the five adrenalectomized dogs subjected to haemorrhagic hypotension after phenoxybenzamine treatment.

TABLE III

PLASMA ADRENALINE AND NORADRENALINE CONCENTRATION IN ADRENALECTOMIZED DOGS DURING HAEMORRHAGIC HYPOTENSION

The concentrations of adrenaline (A) and noradrenaline (N) are expressed in μg./l. Paired values in lowest line represent mean arterial blood pressure (B.P.) before and after withdrawal of each sample.

Expt. No.	0 Min.	60 Min.	120 Min.	135 Min.	150 Min.
	A N	A N	A N	A N	A N
1	0·00 0·25	0·00 0·31	0·00 0·43	0·01 0·60	
2	0·00 0·15	0·00 0·21	0·00 0·22	0·06 0·40	0·13 0·43
3	0·14 0·16	0·09 0·22	0·19 0·45	0·38 0·70	
Mean ..	0·05 0·19	0·03 0·25	0·06 0·37	0·15 0·57	
B.P. ..	98 92	100 68	78 59	52 27	23

TABLE IV

TOTAL BLOOD VOLUME WITHDRAWN BEFORE CARDIAC ARREST IN VARIOUS STUDIES

	Mean (ml./kg.)	Range (ml./kg.)
8 normal dogs (Millar and Benfey, 1958) ..	58·9	46·8–69·6
8 normal dogs given phenoxybenzamine ..	38·7	9·3–51·9
3 adrenalectomized dogs	54·5	50·0–58·3
5 adrenalectomized dogs given phenoxybenzamine	44·1	31·1–58·3

Table IV illustrates the blood loss sustained in the experiments described; also included are results obtained previously by inducing graded haemorrhage in normal, untreated dogs (Millar and Benfey, 1958). The previous administration of phenoxybenzamine appeared to reduce the amount of blood which could be withdrawn before final circulatory collapse developed.

DISCUSSION

The results show that phenoxybenzamine administration in dogs led to marked increases in plasma adrenaline and noradrenaline concentration, which supports previous findings that the administration of phenoxybenzamine to dogs anaesthetized with pentobarbitone greatly augmented the urinary excretion of adrenaline and noradrenaline (Benfey, Ledoux, and Melville, 1958).

The absence of any significant rise in arterial adrenaline concentration in adrenalectomized dogs indicates that phenoxybenzamine induced liberation of adrenaline from the adrenal gland. Three adrenalectomized animals showed a gradual rise in noradrenaline concentration after administration of phenoxybenzamine, which suggests that the increased concentration of noradrenaline after phenoxybenzamine may be due partly to an extra-adrenal component.

The increased concentrations of adrenaline and noradrenaline when circulatory collapse due to

haemorrhage was imminent in the eight dogs treated with phenoxybenzamine were not significantly different from the terminal concentrations estimated previously in eight untreated dogs (Millar and Benfey, 1958). The prior administration of phenoxybenzamine did not appear to alter circulating plasma levels of adrenaline and noradrenaline at the stage of *severe* haemorrhagic shock in normal dogs.

However, in the adrenalectomized dogs treated with phenoxybenzamine, rises in arterial noradrenaline concentration could be measured when circulatory collapse was imminent as a result of haemorrhage which were significantly greater than those observed in adrenalectomized animals not treated with phenoxybenzamine. It is apparent that strong sympathetic stimulation induced by severe haemorrhage leads to higher plasma noradrenaline concentrations in adrenalectomized animals following the application of phenoxybenzamine. This could support the observations of Brown and Gillespie (1957) on the noradrenaline concentrations in splenic venous blood following splenic nerve stimulation in cats, when the amount of noradrenaline found was greater following treatment with phenoxybenzamine.

We wish to thank Prof. K. I. Melville for his encouragement and for providing facilities for undertaking these studies.

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A METHOD FOR MEASURING THE ACTIVITY OF COMPOUNDS WITH AN ACTIVITY LIKE VITAMIN K AGAINST INDIRECT ANTICOAGULANTS IN RATS

BY

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A method for the estimation of the activity of compounds like vitamin K using rats treated with an indirect anticoagulant is described. The assay measured the speed of onset and magnitude of the antidotal effect. Under the conditions employed, only vitamin K_1 produced a significant response. Vitamin K_3 and its water-soluble derivatives were inactive. Both the magnitude and the speed of onset of the antidotal effect of vitamin K_1 were found to depend on the extent to which it was in solution. The assay gave results of fair accuracy and reproducibility, which permitted a full statistical analysis and provided an estimate of error.

The biological assay of compounds with vitamin K activity in the vitamin-deficient chick mainly estimates the dietary activity; it does not give an indication of the ability to reverse the hypoprothrombopaenia caused by indirect anticoagulants of the dicoumarol type. For example, in the chick assay vitamin K_3 (2-methyl-1:4-naphthoquinone) is more active than vitamin K_1 (2-methyl-3-phytyl-1:4-naphthoquinone), whereas as an antidote against anticoagulant substances such as dicoumarol vitamin K_1 is more active and shows a more rapid onset of action. Almquist (1954) has made a detailed review of the factors involved in the biological estimation of vitamin K active compounds.

The assay reported here measures the effect of vitamin K active compounds in the rat made hypoprothrombopaenic by giving an indirect anticoagulant, and therefore gives a more specific estimate of the antidotal activity than the chick assay. Under the conditions of the assay, which allow the determination of the magnitude and speed of onset of antidotal effect, only vitamin K_1 has been found to possess activity, while vitamin K_3 and its water soluble derivatives were found to be inactive.

METHODS

Preparation of Animals.—Male rats weighing from 175 to 225 g. were given an oral dose of 0.5 g. of warfarin (3-[α-acetonylbenzyl]-4-hydroxycoumarin) mixed with 5 g./animal/day of feed (hulled oats) for 2 days. The animals were fed at 4 p.m. and the assay performed 17 to 20 hr. after the second dose, when the prothrombin time (" bedside method ": see below) had increased from a normal value of 30 sec. to over 300 sec. Special feed dishes were used to prevent scattering of feed, and animals which failed to eat all their ration were rejected from the assay. Preliminary experiments showed that the addition of the anticoagulant to the feed gave a more uniform increase of the prothrombin time than intraperitoneal or intravenous injection.

Withdrawal of Blood Samples.—Blood samples were drawn from a lateral tail vein. The rats were restrained either by being wrapped tightly in a towel or by being lightly anaesthetized with ethyl ether. To draw blood samples consistently by tail vein puncture certain technical details had to be carefully observed. The tail was warmed in water at 45 to 50° with gentle massaging for about 1 min. or until the veins were well dilated. Too high a temperature caused collapse of the veins. Blood was drawn with a 27 gauge ½ in. needle and a 0.5 ml. tuberculin syringe. The plunger of the syringe fitted the barrel tightly. If necessary, stopcock grease was used to obtain a close fit. The rate of drawing the blood was adjusted to the rate of flow in order to avoid collapse of the vein. All glassware was scrupulously clean, particularly the needle, since the presence of any trace of material with thromboplastic activity may induce clotting due to the slow rate at which the blood is drawn. Needles were treated with Arquad (Jaques, 1955) and syringes were coated with silicone (Jaques, 1955). Many investigators have been discouraged from using the technique of drawing blood from the tail vein of the rat because of the difficulties encountered before

skill is acquired. Attention to the details listed above may avoid some of these difficulties.

For the "bedside method," blood was drawn without anticoagulant. For the proconvertin method, exactly 0.1 ml. of blood was drawn into 0.4 ml. of a buffered citrate solution (Dyggve and Lund, 1954). The sample was placed in a plastic test tube (75 × 10 mm.) and centrifuged at 2,500 rev./min. for 10 min. The plasma obtained thus will be referred to as diluted citrated plasma.

Prothrombin Time Determination: Bedside Method (A).—This was a modification of the "bedside method" of Schwager and Jaques (1949). One drop of rabbit brain thromboplastin suspension and one drop of freshly drawn whole blood were placed separately on a 1.5 in. watch glass. As the two drops were mixed with a needle, a stopwatch was started and the mixture was stirred gently until a distinct clot formed at the tip of the needle.

Method for the Specific Determination of Proconvertin (B).—This was a modification of the micromethod of Dyggve and Lund (1954) for the specific determination of proconvertin. The test plasma consisted of 0.1 ml. of diluted citrated plasma in a silicone coated test tube (75 × 10 mm.) to which was added 0.1 ml. of sodium citrate solution, 0.2 ml. proconvertin-free bovine plasma and 0.2 ml. rabbit brain thromboplastin. The determination was carried out in a water bath at 37° and was started by adding 0.1 ml. $CaCl_2$ of optimal concentration: this was determined for each batch of proconvertin-free bovine plasma. The proconvertin-free bovine plasma was prepared according to the method of Biggs and MacFarlane (1957).

Vitamin K_1 Preparations.—The vitamin K_1 for the preparation of the standard was obtained from commercial sources and purified by column chromatography (Binkley, MacCorquodale, Thayer, and Doisy, 1939). The vitamin was dissolved in benzene, absorbed on Folin's Permutite and eluted with benzene/petrol ether (20%/80% v/v); small amounts of impurities were retained on the column. The eluate was concentrated *in vacuo* at 30° and the vitamin stored in closed ampoules protected from light.

Vitamin K (40 mg.) was dissolved in Tween 80 (0.5 ml. polyoxyethylene sorbitan mono-oleate, Atlas Powder Co.). The vitamin was mixed carefully with Tween 80 to give a homogeneous paste and water was added in small quantities, the mixture being worked carefully into a homogeneous suspension after each addition and finally made up to 10 ml. The final suspension was completely clear. If the vitamin was not mixed carefully with Tween 80 before the addition of water, or if all the water was added at once, a turbid suspension resulted which possessed considerably less activity. Tween 80 at the concentration used did not change the prothrombin time.

The other two preparations tested were vitamin K_1 suspensions at present marketed under proprietary names.

Results

Preliminary experiments indicated that the vitamin K_1 standard dissolved in Tween 80 could be sensitively assayed between 10 and 80 μg./animal. It produced an optimal antidotal effect after 30 min. Tables I and II show the response to graded doses after 30 min. when the determination of the prothrombin time was carried out by the bedside method (method A) and the proconvertin method (method B) respectively.

In the sensitive assay range, the reciprocal of the prothrombin time was found to be a rectilinear function of the logarithm of the dose, and the data were analysed after this transformation. The results of the standard analysis of variance are shown in Tables I and II. For both methods the mean square due to linear regression was highly significant, and that due to deviation from linear regression was not significant when tested against the residual mean square due to deviation within doses. The assay using method B was carried out on two consecutive days. The mean square due to variations between days was just significant at the 5% level of probability (Table II).

Figs. 1 and 2 show the log dose/response curves fitted by the method of least squares with the exact 95% confidence limits for the estimated most probable log dose.

TABLE I

ANTIDOTAL ACTIVITY OF VITAMIN K. PROTHROMBIN TIME OF RATS PRETREATED WITH WARFARIN (METHOD A)

Prothrombin times (sec.) for each animal.

	Vitamin K_1 (μg./Animal)			
	10	20	40	80
	70	59	48	37
	95	65	38	40
	113	108	63	38
	226	219	86	35
	380	136	44	
	200	102	49	
Mean	187·3	115·0	54·6	37·5

Analysis of Variance

Source of Variance	Sum Sq.	d.f.	Mean Sq.	F	P
Regression ..	1,090·4	1	1,090·4	55·7	< 0·001
Deviations from linearity ..	42·4	2	21·2	1·08	> 0·20
Between doses ..	1,132·8	3			
Residual error ..	53·2	18	19·6		
Total ..	1,486·0	21			

b: $-22{\cdot}25 \pm 2{\cdot}94$ (s.e.). λ: 0·199.

b is the slope of the regression line and $\lambda = \sqrt{\frac{\text{Mean Square Error}}{b}}$

TABLE II
ANTIDOTAL ACTIVITY OF VITAMIN K_1. PROTHROMBIN TIME OF RATS PRETREATED WITH WARFARIN (METHOD B)

Prothrombin times (sec.) for each animal.

	Vitamin K_1 (μg./Animal)			
	10	20	40	80
1st day ..	360 193 346 295	232 240 203	225 127 123 130	99 112 87
2nd day ..	515 324 483	240 201 112 185	88 119 119	80 113 93 94
Mean ..	359	202	133	97

Analysis of Variance

Sources of Variance	Sum Sq.	d.f.	Mean Sq.	F	P
Regression	219·249366	1	219·249366	172	< 0·001
Deviations from linearity	0·225816	2	0·112908	12·195	< 0·10 > 0·05
Between doses ..	219·475182	3			
Between days ..	8·240553	1	8·240	5·95	< 0·05 > 0·01
Residual error ..	31·887376	23	1·386408		
Total ..	259·60311	27			

b: $-8·31 \pm 0·66$ (s.e.). λ : 0·143.

TABLE III
ASSAYS OF ANTIDOTAL ACTIVITY OF VITAMIN K_1 (METHOD B)

Date	2/57	4/57	1/58	1/58	2/58
No. animals ..	15	28	12	15	12
,, dose levels ..	3	4	3	3	3
Slope (b) ± s.e. ..	−17·04 ±4·43	−8·31 ±0·66	−8·56 ±1·46	−11·61 ±2·12	−10·06 ±2·26
λ	0·248	0·143	0·134	0·173	0·184

When the dose is estimated from the curve for method A (Fig. 1) an estimated dose of 10 μg. does not differ significantly from an estimated dose of 20 μg., but the differences between the estimations of the other three dose levels are significant ($P<0.05$). For method B, the differences between all four dose levels are significant ($P<0.05$).

Technically, method B is the more convenient, since plasma samples can be set aside for later determinations. In method A, the prothrombin time determinations have to be carried out immediately, an inconvenience when the prothrombin times tend to be long and many samples have to be taken at specified intervals.

Table III summarizes five assays using method B and shows that the results are reproducible over long periods of time.

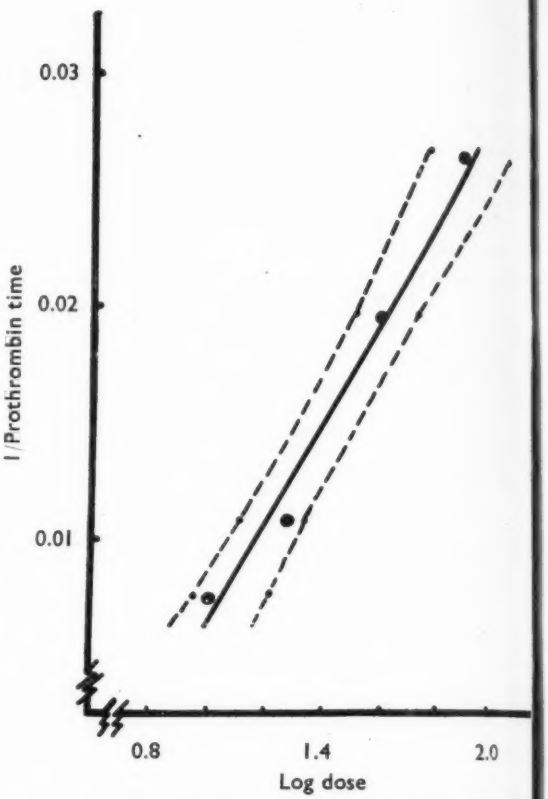

FIG. 1.—Log dose/response line and 95% confidence limits for vitamin K_1 standard determined by method A. Log dose in μg./animal.

The assay has been used to estimate the antidotal activity of two proprietary colloidal solutions of vitamin K_1. Preliminary experiments showed that preparation 1 produced no effect after 30 min., a slight effect after 1 hr., and an optimal antidotal effect only after 2 hr. Preparation 2 showed some activity after 30 min. and optimal antidotal activity after 1 hr. The potency of the two preparations has been estimated against the standard in 4-point parallel line assays. The responses to 20 and 80 μg./animal of the standard after 30 min. and to 100 and 400 μg./animal of the test preparations are shown in Tables IV and V respectively. The effect of preparation 1 was measured after 2 hr. and that of preparation 2 after 1 hr. The standard analysis of variance for a 4-point parallel line assay showed that, for both assays, the mean squares due to regression were highly significant, whereas those due to deviation from parallelism are not significant (Tables IV and V). The relative potencies of preparations 1 and 2 in terms of the standard are 0.250 and 0.256

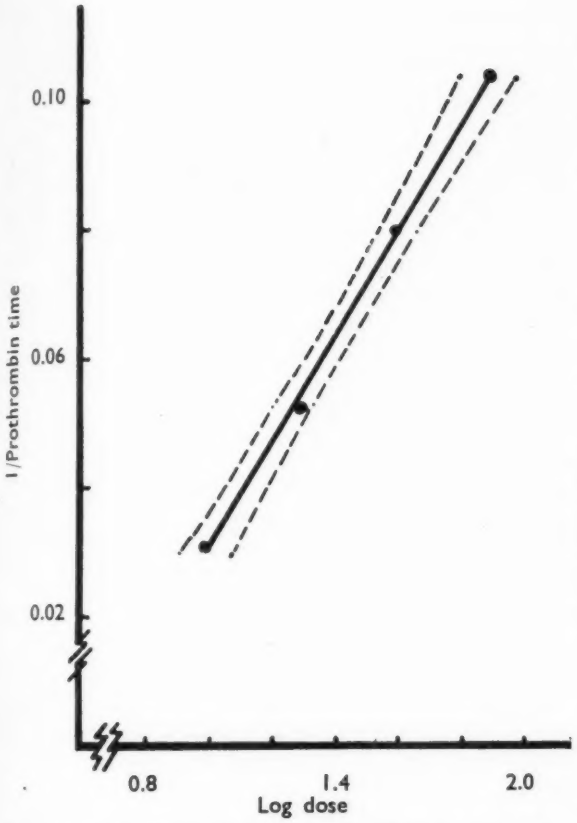

FIG. 2.—Log dose response line and 95% confidence limits for vitamin K_1 standard determined by method B. Log dose in μg./animal.

respectively; the differences are statistically significant for both preparations.

DISCUSSION

The antidotal activity of vitamin K active compounds against indirect anticoagulant drugs is determined by the magnitude and speed of onset. Of these two factors, the speed of onset is important when vitamin K active compounds are used for the correction of excessively prolonged prothrombin times, for the arrest of haemorrhage due to overdosage of anticoagulant drugs, or for the preparation of a patient under anticoagulant treatment for surgery, when it is desirable to return the prothrombin time to normal as rapidly as possible. Although it has been recognized clinically that the onset of the antidotal effect of vitamin K_1 is more rapid than that of vitamin K_3 and its water-soluble derivatives, the use of the chick assay to estimate the potency of vitamin K active compounds has tended to obscure these differences. The assay described here measures the antidotal activity under experimental conditions which approximate more closely to those encountered clinically and therefore gives a more direct estimate of the relative potency. Although the assay has been designed to estimate antidotal activity against anticoagulant drugs

TABLE IV

COMPARISON OF ANTIDOTAL ACTIVITIES OF VITAMIN K_1 PREPARATION 1 AND VITAMIN K_1 STANDARD (METHOD B)

Five animals/treatment. Prothrombin times in sec.

Doses in μg./animal:	Standard		Preparation 1	
	20	80	100	400
	232	99	168	92
	240	112	117	64
	203	87	232	97
	240	80	167	87
	201	113	169	66
Mean	223	98	171	81

Analysis of Variance

Source of Variance	Sum Sq.	d.f.	Mean Sq.	F	P
Preparations ..	19·523	1	10·523	6·901	<0·05
Regression ..	192·572	1	192·572	68·070	<0·001
Parallelism ..	9·585	1	0·585	4·948	>0·20
Between doses ..	212·680	3			
Residual error ..	45·259	16	2·829		
Total ..	257·939	19			

b: −20·62. λ: 0·081. Log rel. potency (M)±s.e. = −0·603±0·109. Rel. potency (R) = 0·250. 95% limits of R: 0·189−0·337.

TABLE V

COMPARISON OF ANTIDOTAL ACTIVITIES OF VITAMIN K_1 PREPARATION 2 AND VITAMIN K_1 STANDARD (METHOD B)

Six animals/treatment. Prothrombin times in sec.

Doses in μg./animal:	Standard		Preparation 2	
	20	80	100	400
	189·0	48·0	90·9	47·5
	122·0	44·0	84·3	56·4
	157·8	51·0	112·6	26·3
	106·0	39·5	91·9	46·3
	116·0	47·6	111·7	40·1
	92·2	65·8	129·1	52·6
Mean	160·5	49·3	103·4	45·9

Analysis of Variance

Sources of Variance	Sum Sq.	d.f.	Mean Sq.	F	P
Preparations ..	32·900	1	32·900	1·795	>0·10
Regression ..	1,051·521	1	1,051·521	57·391	<0·001
Parallelism ..	1·995	1	1·995	9·183	>0·20
Between doses	1,086·416	3			
Residual error	366·431	20	18·322		
Total ..	1,452·847	23			

b: −21·99. λ: 0·195. Log rel. potency (M)±s.e. = −0·592 ±0·078. Rel. potency (R) = 0·256. 95% limits of R: 0·214–0·306.

such as coumarin, it could also be used to estimate vitamin K activity in biological material in place of the chick assay. It is likely to be found more convenient in laboratories which lack facilities for work with the chick or want to avoid the preparation of vitamin K deficient diets.

Previous attempts to use the rat for the bioassay of vitamin K active compounds have not been entirely satisfactory because of the difficulties encountered in making the animal deficient by dietary means (Greaves, 1939). Special procedures (such as ligation of the bile duct) which interfere with the absorption of the vitamin from the intestine have been tried (Flynn and Warner, 1940). Quick and Stefanini (1948) showed that the chick pretreated with dicoumarol can be used for the estimation of vitamin K.

Under the conditions of the assay, which involved a significant reduction of the prothrombin time within 2 hr., vitamin K_3 and its water-soluble derivatives failed to show any activity even when given in such large doses that they produced toxic effects. This observation differs somewhat from the results of Dam and Søndergaard (1953), who observed that, in the vitamin K deficient chick, vitamin K_3 caused a decrease of the elevated prothrombin time during this interval. In a vitamin K deficient animal, the primary defect is in the synthesis or availability of the vitamin. If vitamin K_3 served as a precursor for a more complex derivative, the rate of synthesis of this derivative in the vitamin deficient animal might be sufficient to correct the coagulation defect within 2 hr. In contrast, the mode of action of the indirect anticoagulant drugs is generally believed to be due to competitive inhibition with vitamin K. To overcome this inhibition, larger amounts of the active derivative, in excess of the rate of synthesis, may be necessary. The more complex vitamin K_1, on the other hand, may act directly or with only slight structural changes. The difference in activity of the three vitamin K_1 preparations tested appears to be due to the extent to which the fat-soluble, water-insoluble vitamins were in solution. Dam and Søndergaard (1953) also used Tween 80 for dissolving vitamin K_1 and showed that this preparation had a very rapid onset of action in the vitamin K deficient chick. Although the three preparations tested differed significantly in activity, the parallelism of the dose/response curves indicated that their mode of action was nevertheless the same.

We wish to express our thanks to Miss L. Duncan from our laboratory for her valuable assistance during the course of this work, which was supported by a grant from the National Research Council of Canada.

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THE MECHANISM OF THE INHIBITION BY CARBONIC ACID OF THE SMOOTH MUSCLE CONTRACTION PRODUCED BY HISTAMINE AND OXYTOCIN

BY

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Carbonic acid inhibited the stimulating action of histamine on guinea-pig intestine and of oxytocin on the uterus of many animal species. This effect was relatively specific. Under the same conditions the actions of acetylcholine, KCl, adrenaline (on rabbit uterus), and ergot alkaloids were not inhibited. The electrical excitability of smooth muscle was similarly not affected. The inhibitory action of carbonic acid is directly proportional to its concentration in the medium in which the isolated organ is maintained. The study of the activity of histamine and oxytocin at different *p*H in a medium free of $NaHCO_3$ and buffered with a mixture of sodium maleate and maleic acid suggested that the inhibitory action exerted by carbonic acid was specific and independent of the simultaneous modifications of the hydrogen ion concentration. The mechanism of these phenomena is discussed.

Evidence has been provided recently that carbon dioxide inhibits the excitability of peripheral nerves by a specific action (Lorente de Nó, 1947; Monnier, 1952; Coraboeuf, 1951, 1954). On the other hand, as early as 1923 Lovatt-Evans and Underhill had shown that CO_2 exerts an inhibitory action on the contractions of isolated intestine produced by different substances. Later, Garan (1938) showed that the activity of histamine on isolated guinea-pig intestine was a function of the partial pressure of CO_2 and attributed the effect to the formation of a carbamate compound, supposedly inactive, between histamine and CO_2. The conclusions of Garan (1938) were disproved by Kiese (1940), who also observed that CO_2 diminished the response of the intestine to histamine, although the action of acetylcholine was but little influenced and that of pilocarpine was enhanced. By studying the relationship between *p*H and the activity of histamine, Kiese (1940) concluded that the change in the polarity of the histamine molecule by modifications of *p*H could not satisfactorily explain the effect observed.

More recently, Halpern (1956a and b) studied the action of CO_2 on the contractions of the isolated guinea-pig ileum produced by histamine, acetylcholine, KCl and the antigen-antibody reaction. An inhibitory effect was found on contractions produced by histamine and in anaphylactic reactions, but under the same conditions the effect of acetylcholine remained unchanged or was increased. The effect of CO_2 was shown to be completely reversible. It could not be attributed to tissue anoxia, because it was produced by gas mixtures containing 20% CO_2 and 80% O_2: the partial pressure of oxygen was therefore higher than when air was used to oxygenate the medium.

Halpern, Mayer, and Bugnard (1956) have demonstrated that CO_2 also inhibits the action of oxytocin on the uterus of the rabbit, guinea-pig and rat. Here, too, the effect was specific for oxytocin and did not apply to the action of other substances which cause the uterus to contract.

The aim of the present work was to discover how carbon dioxide inhibits the action of histamine and oxytocin on smooth muscle. The results suggest that carbon dioxide modifies the reactivity of smooth muscle to these substances by a specific action which is not dependent upon the changes it produces in the *p*H of the medium.

METHODS

The investigations were performed either with isolated guinea-pig intestine or isolated uteri from guinea-pigs, rabbits, and rats. The organs were taken

immediately after killing the animals by carotid artery section. Strips of 2 to 3 cm. were cut off and immersed in oxygenated Tyrode solution at 37° containing the following ingredients in 100 ml.: NaCl 0.8 g.; $CaCl_2$ 0.01 g.; KCl 0.02 g.; $MgCl_2$ 0.01 g.; NaH_2PO_4 0.005 g.; $NaHCO_3$ 0.1 g.; dextrose 0.1 g.; atropine 3 μg. When the concentration of $NaHCO_3$ was changed, a modification was made in the NaCl concentration to maintain the same molarity of the solution. In one series of experiments, a solution without $NaHCO_3$ was used and the *p*H was adjusted to the desired value with a buffer of sodium maleate and maleic acid. The composition of Tyrode solution was modified in this instance by replacing the $NaHCO_3$ by 0.07 g. of maleic acid (6 mM). The *p*H was then adjusted with N-NaOH (approximately 1.1 ml./l. to obtain *p*H=7.5). The solutions of histamine dihydrochloride, acetylcholine chloride, oxytocin, etc., were prepared in Tyrode solution at *p*H 7.4. The volume added to the bath was 0.1 to 0.5 ml. The total volume of the vessel in which the organ was immersed was 50 ml. The contractions were recorded on smoked paper, by frontal inscription, employing a magnification of about 20 times.

Electrical Stimulation.—The technique described by Paton (1956) was used, using the electronic stimulator of Ead (1951). The anode was platinum wire, 4 to 5 cm. long, introduced in the lumen of the intestine. The cathode was a similar wire in the solution. Stimulation was coaxial. A diagram of the apparatus is seen in Fig. 1.

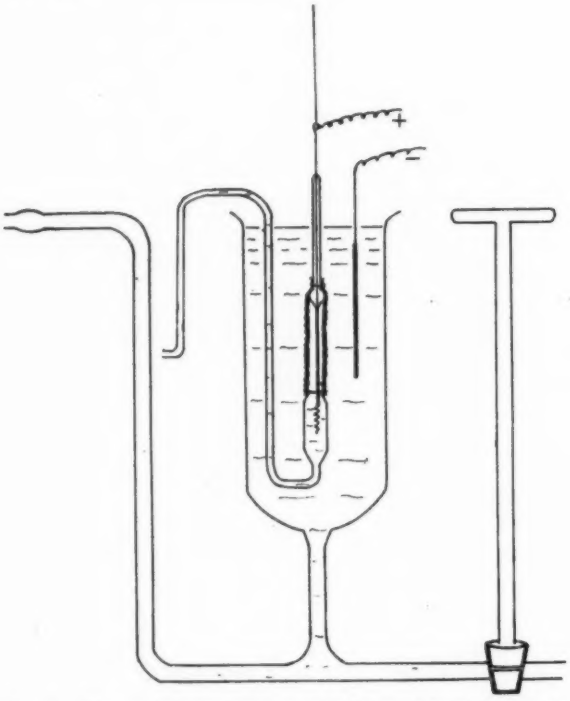

FIG. 1.—Diagram of apparatus for electrical coaxial stimulation of a smooth muscle tube.

Rectangular pulses of 20 msec. duration every 10 sec. were used to stimulate the gut. 20 V usually produced supramaximal stimulation.

pH Measurement.—The *p*H of the solution was measured instantaneously with a laboratory type *p*H meter (Cambridge Instrument Co.). The glass and calomel electrodes were immersed in the vessel in which the organ was studied.

Introduction of Gas Mixtures.—Air, oxygen, CO_2, or mixtures of CO_2 and O_2 were bubbled through the solution in each experiment. With air or oxygen, the *p*H of the Tyrode solution, initially about 7.5, slowly increased to 8.2 or even 8.5. A mixture rich in CO_2 produced a diminution of the *p*H. A typical experiment is illustrated in Fig. 2. By simultaneously bubbling air and a mixture of CO_2 and O_2, at different rates, the desired *p*H could be maintained for long enough to carry out the experiments. The H_2CO_3 content of the solution was always calculated from the measured value of *p*H according to the Henderson-Hasselbach equation:

$$pH = pK + \log \frac{(HCO_3^-)}{(H_2CO_3)}$$

where *p*K is the negative logarithm of the dissociation constant of carbonic acid. The value of *p*K, at 37°, is 6.31. In our calculations no allowance was made for activity coefficients and (HCO_3^-) was assumed to be equal to the $NaHCO_3$ content of the solution employed.

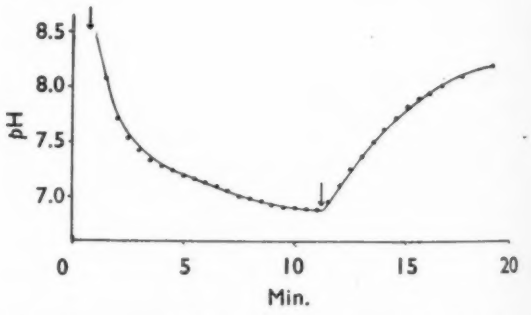

FIG. 2.—Modification of the *p*H of the normal Tyrode solution produced by the bubbling with 20% CO_2 from first to second arrow. At second arrow bubbling with air was commenced.

RESULTS

Relationship Between H_2CO_3 Concentration and Reactivity of the Isolated Guinea-pig Intestine to Histamine

Two methods were used to evaluate the effect of the H_2CO_3 on the action of histamine. With the first method, the height of the contraction of the isolated guinea-pig intestine produced by a fixed dose of histamine at different H_2CO_3 concentrations was determined. The results are shown in Fig. 3.

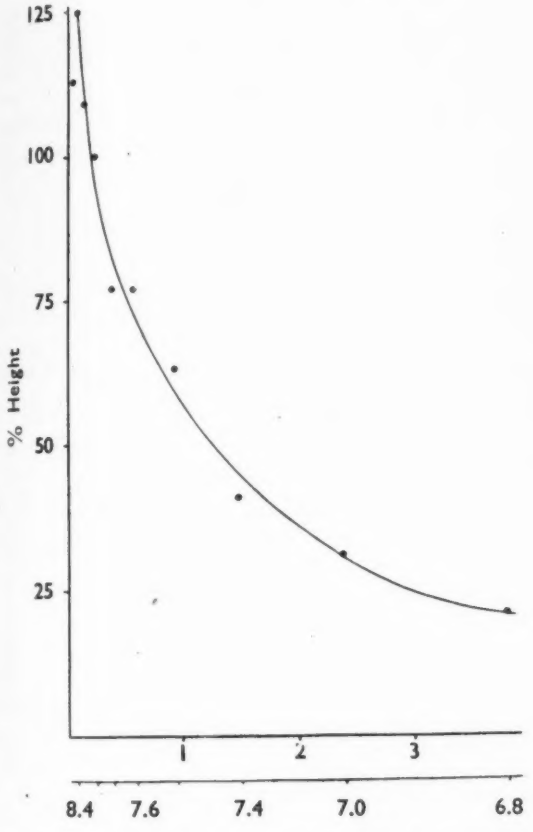

FIG. 3.—The height of the contraction of the isolated guinea-pig intestine preparation produced by a fixed quantity of histamine at various H_2CO_3 concentration in normal Tyrode solution expressed as % of that at *p*H 8·0 to 8·1 given the arbitrary value of 100. The *p*H values are indicated on the lower abscissa, while the H_2CO_3 concentration (mM/l.) is shown on the upper abscissa. Each point was based on at least 5 determinations with each of 4 strips of muscle.

In comparing the different contractions, the height of the one taking place at *p*H 8.0 to 8.1 was taken as 100%, and the heights at other *p*H values were referred to it. In every case, the same dose of histamine was used through the entire experiment and it was chosen so that vigorous but submaximal contractions were obtained. The dose of histamine base varied between 0.015 and 0.030 μg./ml., depending on the particular sensitivity of each intestinal strip.

The results show that the activity of histamine was diminished as the H_2CO_3 concentration was increased (Fig. 3). With a mixture containing 5% CO_2 and 95% O_2, the *p*H attained was 7.3 and the action of histamine was diminished by about 50% of that at *p*H 8.0. With a mixture containing 20% CO_2, equilibrium was reached at *p*H 6.8 and the diminution of activity was 80%.

It is impossible to measure precisely the degree of inhibition by this method because there was no direct relation between the height of the contraction and the concentration of histamine. Furthermore, at a *p*H of less than 6.8, the activity was so low that measurements could not be continued. To overcome these difficulties the second method was employed. This required the determination of the dose of histamine which produced the same response of the isolated intestine at different H_2CO_3 concentrations. The results in Fig. 4 show that, when equiactive doses of histamine are plotted against the H_2CO_3 concentration, a straight line was obtained. As the quantity of histamine necessary to produce the same effect was the reciprocal of its activity, it can be deduced from the observed relationship that the activity of the histamine was inversely proportional to the concentration of H_2CO_3.

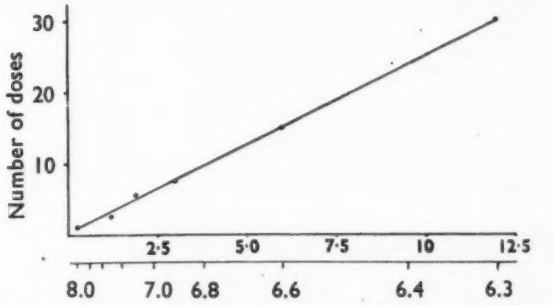

FIG. 4.—Dose of histamine (expressed in terms of the multiple of the dose required at *p*H 8·0) producing the same response of the isolated guinea-pig intestine preparation at various H_2CO_3 concentration, in normal Tyrode solution.

Role of pH

As the action of histamine on smooth muscle was modified by the H_2CO_3 content of the Tyrode solution, it was necessary to find whether this effect was due to changes in the *p*H or was produced specifically by H_2CO_3.

It is impossible to modify the H_2CO_3 concentration of Tyrode solution without simultaneously changing the *p*H, all the other conditions remaining the same. We approached this problem in two ways: (a) by modifying the $NaHCO_3$ of Tyrode solution; (b) by employing a buffer of different composition.

Influence of $NaHCO_3$ Concentration on the Reactivity of the Isolated Guinea-pig Intestine to Histamine and Acetylcholine.—By using Tyrode solutions which contained between 0.25 and 10 g./l. of $NaHCO_3$, it was possible to obtain a wide variation in *p*H with the same concentration of H_2CO_3. When air is bubbled through solutions containing 5 or 10 g./l. $NaHCO_3$, the *p*H reaches

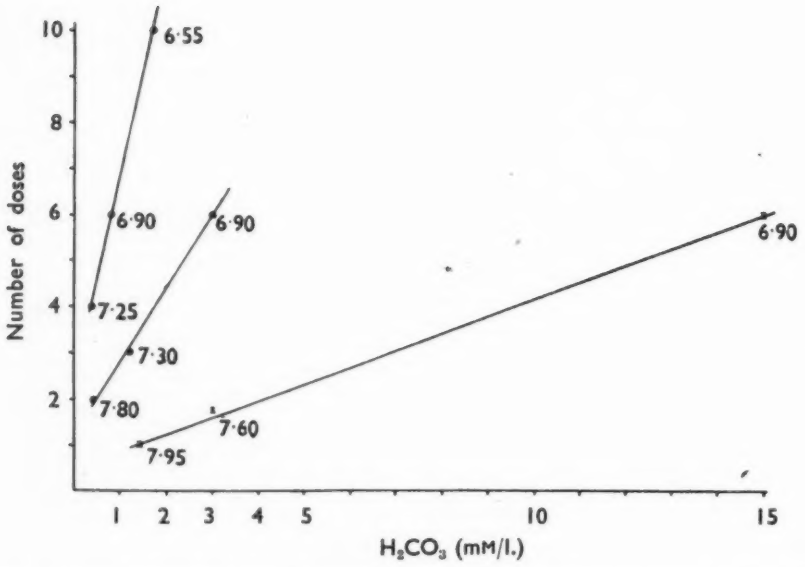

FIG. 5.—Dose of histamine (see Fig. 4) producing the same response of the isolated guinea-pig intestine preparation at various H_2CO_3 concentration and at various $NaHCO_3$ concentration. Upper curve $NaHCO_3$ 0·25 g./l.; middle curve $NaHCO_3$ 1 g./l.; lower curve $NaHCO_3$ 5 g./l. The numerals on the graph indicate the *p*H value at each point.

9 or more and the medium becomes quite unfavourable for the organ.

The results in Fig. 5 show that the effect of histamine diminished with increasing H_2CO_3 concentration at the same $NaHCO_3$ concentration and increased with increasing $NaHCO_3$ concentration at the same H_2CO_3 concentration. In addition, the effect of histamine on the guinea-pig intestine remained practically the same at constant *p*H and independent of the actual concentration of $NaHCO_3$ and H_2CO_3 (Fig. 6).

It was therefore impossible to decide, in this way, the exact rôle of *p*H.

Experiments with a Different Buffer System.—Many solutions were tried to find a convenient medium, free from $NaHCO_3$. Finally a solution containing maleic acid and sodium maleate proved quite suitable and, in it, organs responded regularly and reversibly for the periods of time essential for quantitative study. This was not so with solutions buffered with substances such as phosphate or citrate, which produced more or less irreversible modifications in the responses of the tissues.

Fig. 7 shows the results obtained when the activity of histamine was examined in the maleic acid/sodium maleate solution at different *p*H values, and, for comparison, the observations upon histamine activity in normal Tyrode solution of which the *p*H was modified by CO_2.

It appeared that the activity of histamine was not greatly affected by changes in *p*H, unless these were produced by increasing the H_2CO_3 concentration. In fact, at *p*H 6.8 the action of histamine was practically unchanged in the maleate medium, while it was very much reduced in normal Tyrode solution. It may be concluded, therefore, that carbonic acid exerts some more or less specific action.

Influence of H_2CO_3 on the Smooth Muscle Contractions Produced by Electrical Stimulation or by Substances Other than Histamine

It was of interest to investigate the specificity of the action of H_2CO_3 on the response to histamine. The results were as follows: The action of a standard dose of acetylcholine in guinea-pig intestine was un-

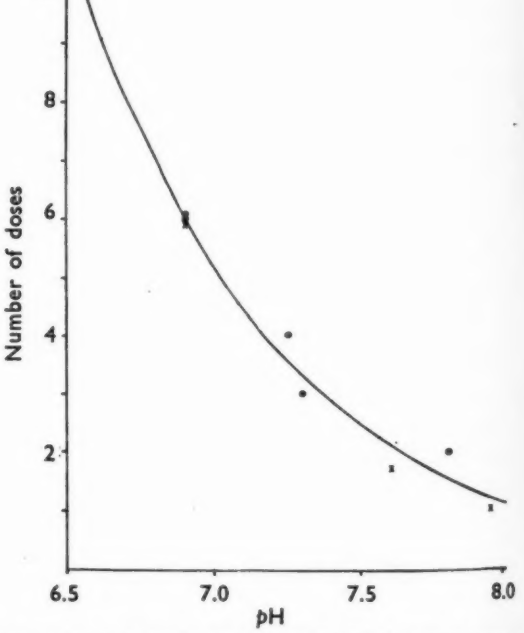

FIG. 6.—Dose of histamine (see Fig. 4) producing the same response of the isolated guinea-pig intestine preparation at different *p*H values, using Tyrode solution with different concentrations of $NaHCO_3$ (O=0.25 g./l.; ●=1 g./l.; X=5 g./l.).

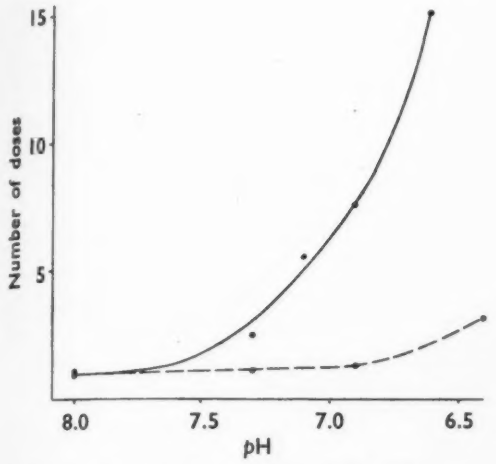

FIG. 7.—Dose of histamine (see Fig. 4) producing the same response of the isolated guinea-pig intestine preparation at different *p*H values. Full line, in normal Tyrode solution (bicarbonate buffer); broken line, in Tyrode solution buffered with sodium maleate and maleic acid.

affected or enhanced by the H_2CO_3 within the limits studied (Fig. 8). The action of KCl was not modified between *p*H 8.1 and 6.9. The contraction of the intestine produced by electrical excitation was not modified by H_2CO_3 between *p*H 8.1 and 6.9 (Fig. 9).

Influence of H_2CO_3 Concentration on the Response of the Isolated Uterus to Oxytocin

Halpern *et al.* (1956) showed that H_2CO_3 can inhibit the action of oxytocin on the isolated uterus as illustrated in Fig. 10. Experiments with KCl, adrenaline (rabbit uterus) and methylergometrine (Methergen) showed that H_2CO_3 only modified the oxytocin response (Fig. 11). The relationship of the activity of oxytocin to *p*H showed that it was greatly inhibited at *p*H 6.8 in Tyrode solution (bicarbonate buffer) but that it was not changed at the same *p*H in the maleate buffered medium (Fig. 12). The observations upon histamine and oxytocin thus resemble each other.

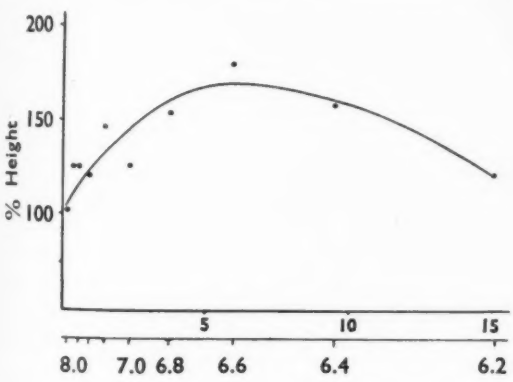

FIG. 8.—Height of the contraction (see Fig. 3) of the isolated guinea-pig intestine preparation produced by a fixed quantity of acetylcholine at different H_2CO_3 concentration, in normal Tyrode solution. Upper abscissa, H_2CO_3 mM/l.; lower abscissa, *p*H.

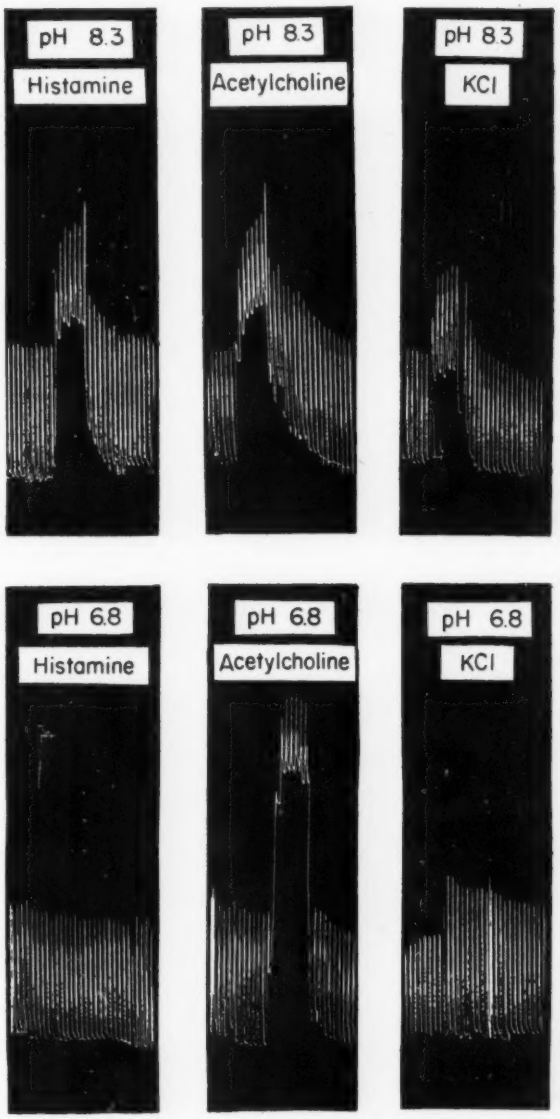

FIG. 9.—Contractions of the isolated guinea-pig ileum preparation in response to periodic electric stimulation. Pulse width, 20 msec.; frequency, 6 min.; 20 V. Upper records: effects of histamine (1.5×10^{-8}), of acetylcholine (0.5×10^{-8}) and KCl (1×10^{-3}) on the responses of the intestine in standard Tyrode solution (buffer, $NaHCO_3$ 1 g./l.), bubbled with pure oxygen. Lower records: effects of identical concentrations of the same substances on the intestine in the same Tyrode solution, the *p*H of which has been lowered to 6·8 by bubbling with a gaseous mixture containing 75% O_2 and 25% CO_2.

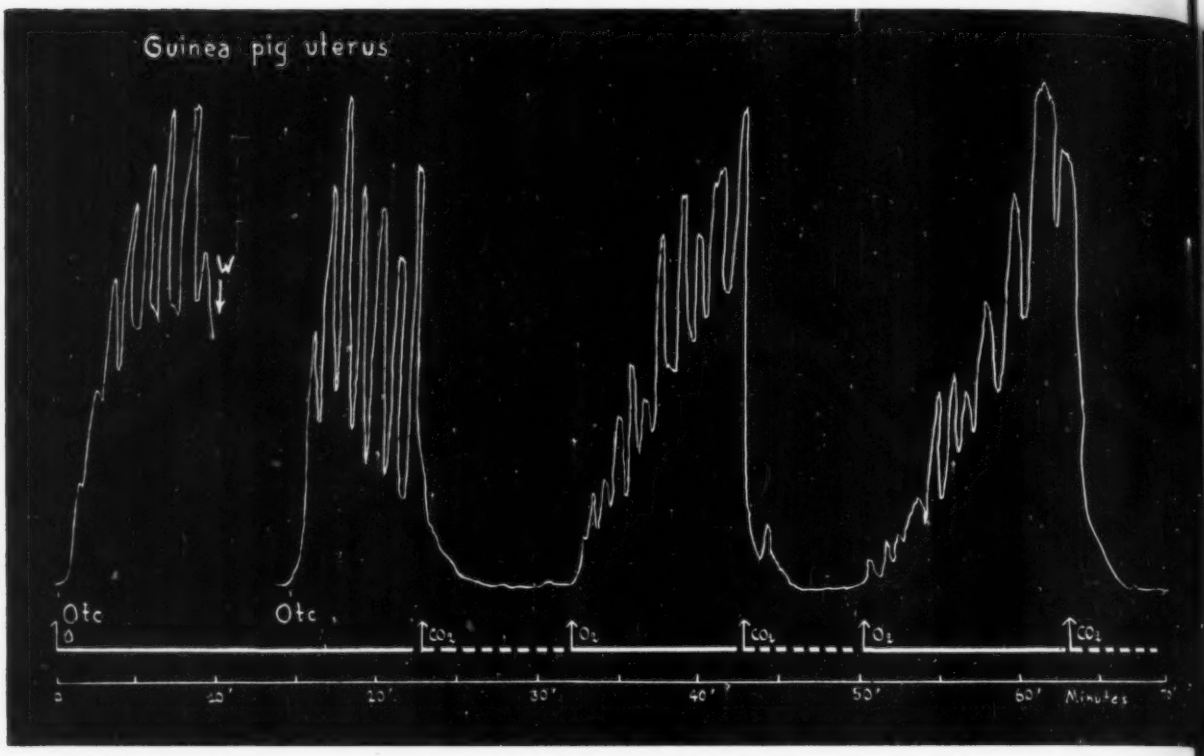

FIG. 10.—Isolated guinea-pig uterus preparation in standard Tyrode solution (buffer, $NaHCO_3$ 1 g./l.). From left to right: Effect of a standard dose of oxytocin (1×10^{-4} I.U.) on the uterus in Tyrode solution oxygenated with pure oxygen. After washing, addition of the same dose of hormone produces a similar effect. Without removing the hormone, a gaseous mixture containing 75% O_2 and 25% CO_2 was substituted for oxygen at the point marked CO_2. At the point O_2, pure oxygen was substituted for the gaseous mixture. The same procedure is repeated twice. **Otc**=Oxytocin; W=wash. Full line=pure oxygen; broken line=gaseous mixture containing 75% O_2 and 25% CO_2. Time, 5 min.

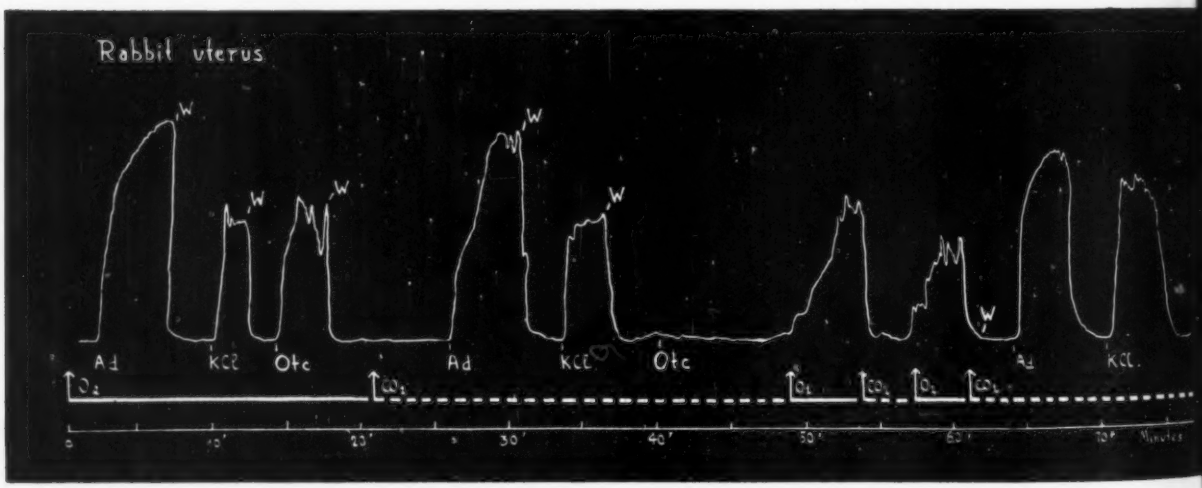

FIG. 11.—Isolated rabbit uterus preparation in standard Tyrode solution (buffer, $NaHCO_3$ 1 g./l.). From left to right: Effects of a standard dose of adrenaline (2×10^{-6}), KCl (5×10^{-4}) and of oxytocin (1×10^{-6} I.U.) on the uterus in Tyrode solution oxygenated with pure oxygen (full line). After washing, a gaseous mixture containing 75% O_2 and 25% CO_2 was substituted at the point CO_2 and the responses to the drugs again tested. Without washing out the oxytocin, pure oxygen was substituted for the gaseous mixture at O_2. **Ad**=adrenaline; **Otc**=oxytocin; W=wash. Full line=pure oxygen; broken line=gaseous mixture containing 75% O_2 and 25% CO_2. Time, 5 min.

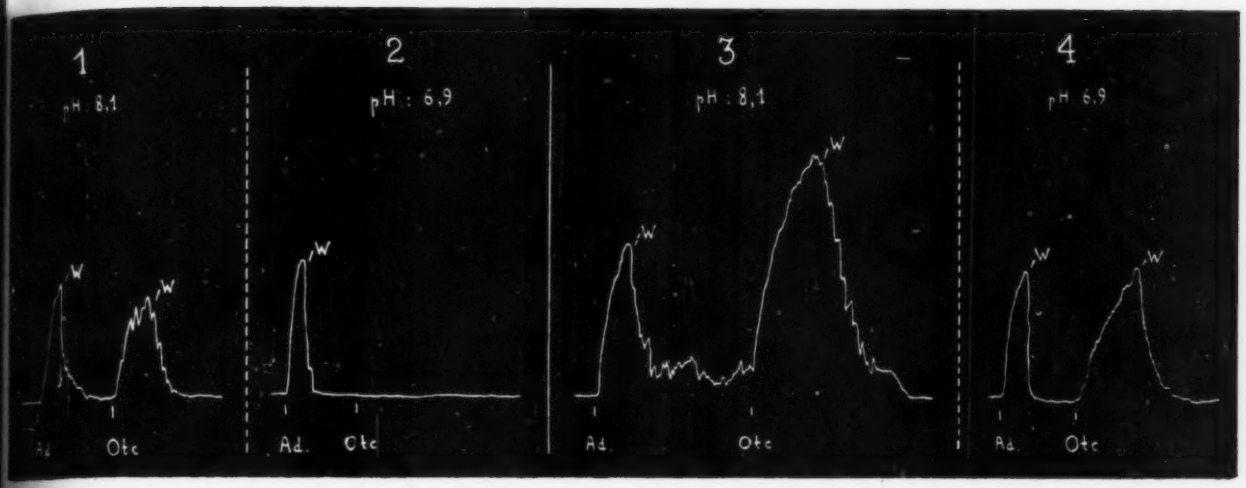

FIG. 12.—Isolated rabbit uterus horn preparation. On the left side (**1** and **2**) in standard Tyrode solution (buffer, $NaHCO_3$ 1 g./l.); on the right side (**3** and **4**) in Tyrode solution buffered with maleic acid/sodium maleate. **Ad**=adrenaline; **Otc**=oxytocin; **W**=wash. In **1**, effects of a standard dose of adrenaline (2×10^{-6}) and of oxytocin (2×10^{-4} I.U.) in normal Tyrode solution oxygenated with pure oxygen. In **2**, a gaseous mixture containing 75% O_2 and 25% CO_2 was substituted for oxygen and the *p*H fell to 6.9. Note response to adrenaline but the absence of response to oxytocin in the same dosages as in **1**. In **3**, Tyrode solution buffered with maleic acid/sodium maleate. Effects of the same doses of adrenaline and of oxytocin. In **4**, the *p*H of the Tyrode solution has been lowered to 6.9 by change of the buffer constituents (maleic acid/sodium maleate) while the medium is oxygenated with pure oxygen and the effects of the same doses of adrenaline and oxytocin are shown.

DISCUSSION

The results indicate that carbonic acid inhibited the action of histamine and oxytocin on smooth muscle. As has been previously reported, the effect was not due to tissue anoxia. It is crucial to know if the inhibition was produced primarily by reduction in the *p*H of the medium due to modifications in the H_2CO_3 concentration. The introduction of CO_2 into Tyrode solution, buffered with $NaHCO_3$, decreased the *p*H, and it seems logical to relate this fact to the inhibitory action. This supposition is supported by the results of experiments at various $NaHCO_3$ concentrations.

But this hypothesis is invalidated by the fact that when the bicarbonate buffer was replaced by a mixture of sodium maleate and maleic acid, the activity of histamine remained the same in spite of the variations of *p*H. In the presence of H_2CO_3, the action of histamine is diminished about 7 times at *p*H 6.8 in comparison with that at *p*H 8.1, yet it is hardly affected in the maleate medium (Fig. 8). This leads to the conclusions that the inhibition of histamine activity was independent of *p*H and that it was related to the presence of carbonic acid and its salt. The same appears to be true for oxytocin. In some experiments (unpublished observations), similar results were obtained employing a bicarbonate-free Tyrode solution buffered with phosphate.

We can then postulate that the inhibition of the action of histamine and oxytocin is not due to the decrease of *p*H, but that it is determined by a qualitative effect of carbonic acid. What is the nature of the effect? The formation of an inactive compound between histamine and H_2CO_3 seems unlikely. Possibly H_2CO_3 modifies the sensitivity of the specific cellular receptors for certain substances.

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COMPARATIVE EFFECTS OF ANALGESICS ON PAIN THRESHOLD, RESPIRATORY FREQUENCY AND GASTROINTESTINAL PROPULSION

BY

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In the rat, the ratio of the analgesic to the respiratory depressant potency was the same for morphine, codeine, diamorphine, methadone, dipipanone, piperidylisomethadone, phenadoxone, dextromoramide, and propoxyphene. The relative respiratory depressant activity of pethidine tended to be less, but the difference was not significant. The ratio of the analgesic dose to the dose preventing transport of a charcoal meal in the rat was about the same for morphine, codeine, pethidine, methadone, phenadoxone, dimethylthiambutene, and propoxyphene; the relative activities of these compounds in inhibiting the peristaltic reflex of the isolated guinea-pig ileum were also similar. However, because of differences in the slopes of regression lines in the charcoal meal test, some compounds (for example, morphine) had a greater effect on gastrointestinal propulsion than others (for example, pethidine) when given at moderate analgesic dose levels.

In studies of the effects of intracisternal morphine in the rat, effects on the spinal reflex of the tail were to some extent dissociated from effects on the threshold for a squeak response. Further, the delaying of transport of a charcoal meal paralleled depression of respiratory rate, and this is evidence for the participation of a central as well as a peripheral action in the effect of morphine on the gastrointestinal tract. The delay in propulsion was reduced by nalorphine and increased by atropine and two general anaesthetic substances, but was unaffected by a number of other pharmacological agents.

The elevation of pain threshold with morphine and allied drugs is commonly accompanied by a reduction in breathing and a delay in gastrointestinal transport (Krueger, Eddy, and Sumwalt, 1941; Reynolds and Randall, 1957). The relationship between the doses causing these effects is known to a limited extent for a few compounds in man, but such comparisons as have been carried out in laboratory animals have often been unsatisfactory as quantitative studies because of inadequate investigation of the dose/response relationships or of failure to compare the pharmacological effects in the same species under strictly comparable conditions. Comparative observations upon the rat are presented in this paper. The compounds examined represent several series of analgesics, with effects resembling those of morphine even to their being antagonized by nalorphine. The comparisons were usually made after subcutaneous or intravenous administration, but morphine was also given intracisternally to see if a central site was important for these effects. The comparative effects of analgesics on the peristaltic reflex of the isolated guinea-pig ileum and the effects of various pharmacological agents on gastrointestinal propulsion in untreated rats and rats treated with morphine are also reported.

METHODS

Analgesia and Respiratory Frequencies. — Pain thresholds estimated by the heat and pressure methods of Green and Young (1951) were measured 30 min. after the injection of the analgesic substances subcutaneously at two or more concentrations into groups of ten or twenty rats aged three to four weeks. Activities relative to that of morphine, tested on the same occasion, were determined from the regression on log dose of the probit of the proportion of rats in the group with pain thresholds at least twice that of a control group injected with saline. Respiratory frequencies were measured immediately following the

pain threshold determinations by recording the pressure changes in a tambour held against the belly of the animal (Green, 1953a). Respiratory depressant potencies were estimated from the regression of frequency on log dose; no significant differences in slopes were detected.

Gastrointestinal Effects.—The charcoal meal test introduced by Macht and Barba-Gose (1931) for examining cathartics and used by Karr (1947) in testing the effects of morphine, methadone and pethidine, was investigated. The rats were prepared in two ways. Those animals referred to as "fasted" were given no solid food for a day but water *ad libitum* up to 1 hr. before the test. The "dieted" rats received a liquid diet of 5% glucose (w/v) in protein hydrolysate and water *ad libitum*, for two days until 1 hr. before the test. Animals were housed in cages with wire mesh bottoms. The test meal, given by stomach tube, was 0.3 or 0.5 ml. of a smooth suspension containing charcoal, wheat flour and water in the proportions by volume of 1:2:6. At stated time intervals afterwards, the rats were killed by a blow on the head and the proportion of the intestine traversed was measured immediately.

Control studies (Fig. 1) showed that the rate of propulsion was most rapid in the first part of the small intestine and was uniform for the first 10 min. It then progressively decreased. The mean proportion of the small intestine traversed in 10 min. was significantly greater in the dieted rats (0.58, s.e. ± 0.018) than in the fasted rats (0.42 ± 0.024). The 10 min. period was chosen as most suitable for studying drug effects. Because of a small day-to-day variation in controls it has been usual to express the proportions of the intestine traversed as percentages of the mean in a concurrent control group as the first step in analysing the results.

Drugs were normally given subcutaneously or intravenously. In the main comparisons, they were injected subcutaneously 20 min. before the charcoal meal as the analgesic effects of these drugs reached a maximum between 20 and 30 min. after subcutaneous injection in young rats. Most of the drugs included in Fig. 2 were compared in a single experiment on groups of ten rats; five animals were given each dose on one day and five on another. The results for codeine and propoxyphene [(±)-4-dimethylamino - 3 - methyl - 1:2 - diphenylbut - 2 - yl propionate] are from separate experiments using groups of six rats.

In the second comparison of morphine, methadone and pethidine, groups of sixteen animals were given morphine sulphate, 0.153, 0.613, 2.5, 10 or 40 mg./kg;

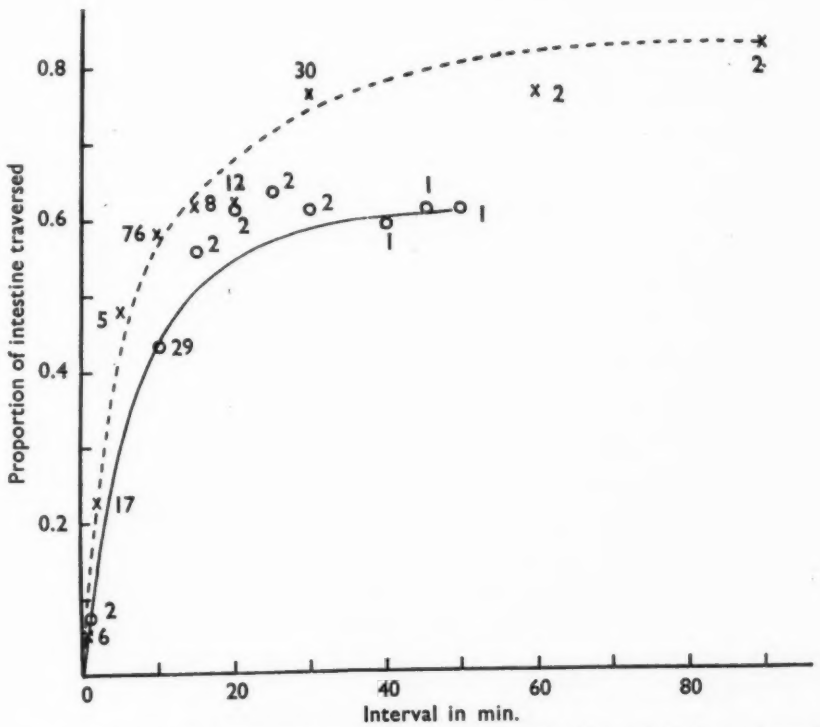

FIG. 1.—The proportion of the small intestine traversed at intervals after administering a charcoal meal in fasted (O—O) and dieted (X - - - X) rats. The number of measurements is indicated by the numeral beside each point.

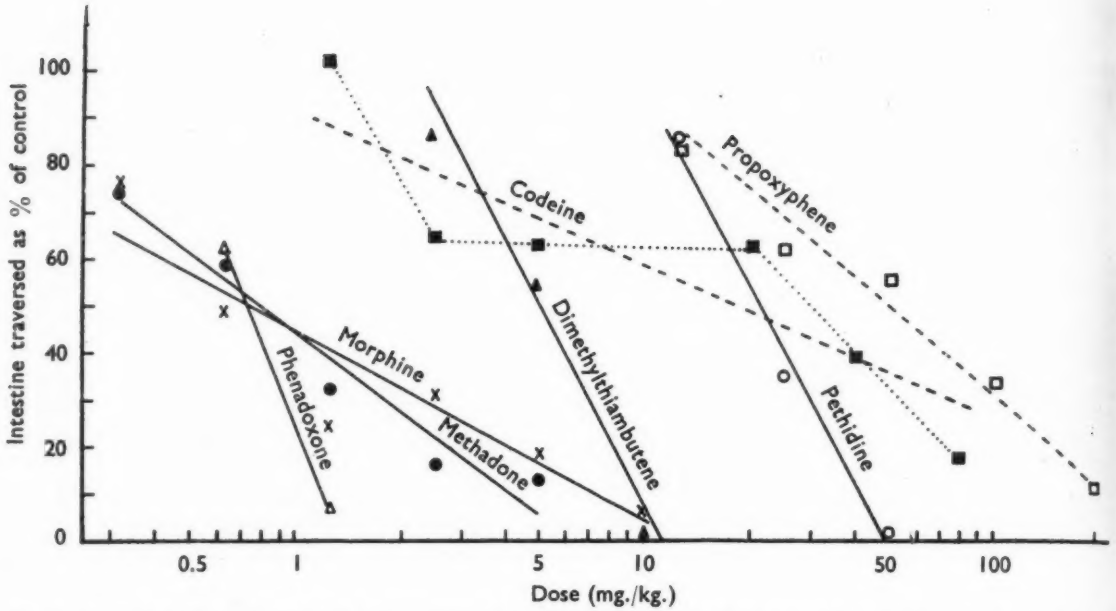

FIG. 2.—The effect of analgesics, injected subcutaneously, on the propulsion of a charcoal meal in rats. The regression on log dose of the proportion of the small intestine traversed by the charcoal in 10 min., as % of the mean in controls. All the compounds except codeine and propoxyphene were compared in the same experiment.

methadone hydrochloride, 0.078, 0.313, 1.25, 5 or 20 mg./kg.; or pethidine hydrochloride, 6.25, 12.5, 25 or 50 mg./kg. The pain thresholds of the animals were measured immediately before they were killed. The rats used in these experiments were three to five weeks old. Saline was injected in controls.

Intracisternal Tests.—The injections were made under short ether anaesthesia using a needle with a bent tip, as described for mice by Lockett and Davis (1958). The needles were 201 gauge with a polythene tube sheath that left only the last 2.5 mm. of the needle exposed. The charcoal meal was administered 20 min. after the morphine, by which time the effects of the ether had largely passed off and the morphine depression was about maximal. Pain thresholds and respiratory frequencies were estimated immediately before killing the rats for measurements of intestinal propulsion. Sixteen rats were used at each dose, four on each of four test occasions. The labels of doses were coded.

Peristaltic Reflex.—The stretch reflex of isolated guinea-pig ileum suspended in oxygenated Tyrode solution at 37° was induced by raising the intraluminal pressure by 2 to 3 cm. water and longitudinal contractions and volume changes recorded by the method of Trendelenburg. The response was determined before and 3 min. after each of a series of increasing doses of the analgesics; the dose interval approximated to $\sqrt{10}$. The log dilutions (pD values) causing slight (±) and nearly complete (+++) inhibition were determined for the longitudinal muscle contractions and the volume change.

RESULTS

The Association of Analgesic and Respiratory Depressant Actions in the Rat

In the rat, the same quantitative relationship between analgesic activity and depression of respiratory frequency was found for morphine and some thiambutene derivatives (Green, 1953a), and by Green and Ward (1956) for morphine and morpheridine (morpholinoethylnorpethidine). Similar comparisons of other drugs are summarized in Table I. (Some of these results were reported to the British Pharmacological Society by Green and Young in 1950.) No significant differences were found between the relative analgesic and respiratory depressant properties of morphine, diamorphine, codeine, pethidine, methadone, phenadoxone, dipipanone, piperidylisomethadone (2-methyl-3:3 diphenyl-1-piperidino-hexan-4-one hydrobromide), dextromoramide and propoxyphene. There was a tendency for the respiratory depressant effect of pethidine to be less ($P=0.07$).

TABLE I

RELATIVE ANALGESIC AND RESPIRATORY DEPRESSANT POTENCIES AND THE PROBABILITIES OF THE RATIOS OF THESE ACTIVITIES BEING UNITY

Activities were usually derived by pooling the estimates from two or more comparative tests; n=the total number of animals given the test substance; an equal number received morphine. The analgesic potencies are from the pressure thresholds for a squeak response; the potencies from heat reaction times were the same.

Drug	n	Relative Activity		A/R	Probability of A/R=1·0
		Analgesia (A)	Respiration (R)		
Morphine sulphate ..	—	1·0	1·0	1·0	—
Codeine phosphate ..	30	0·20	0·22	0·91	>0·5
Diamorphine (base)..	30	7·14	6·49	1·10	>0·5
Pethidine hydrochloride ..	128	0·44	0·31	1·43	0 07
Methadone hydrochloride ..	80	2·48	2·59	0·95	>0·5
Dipipanone hydrochloride ..	80	2·32	2·15	1·08	>0·5
Piperidylisomethadone hydrobromide ..	80	0·93	1·06	0·88	>0·5
Phenadoxone hydrochloride ..	80	5·75	5·15	1·11	>0·5
Dextromoramide (base)	60	13·68	16·72	0·82	>0·5
Propoxyphene hydrochloride ..	110	0·114	0·108	1·05	>0·5

Delay in Gastrointestinal Propulsion

Analgesics Given Subcutaneously.—The effects on the propulsion of a charcoal meal are compared in Table II and Fig. 2. The proportions of the small intestine traversed within 10 min. are expressed as percentages of the mean in the control group. Analysis showed homogeneity of variances, the overall mean value being about 850, but there was a tendency for the variance to decrease at the higher levels of effect. The values were related linearly to log dose for each compound, deviation not being significant at the 5% level. Analysis by the χ^2 test of observations on the five compounds investigated within the same experiment (Table II) showed no significant difference between the slopes for morphine and methadone ($P=0.2$) or between those for pethidine, phenadoxone, and dimethylthiambutene ($P=0.6$), but the slopes for morphine and methadone were significantly less steep ($P<0.001$) than those for the other three compounds. The doses reducing intestinal transport by 50% were calculated and doses stopping the entry of charcoal into the intestine were derived by extrapolation from the dose/response lines. The latter dose of morphine, methadone, pethidine, and dimethylthiambutene was in each instance about four times the corresponding analgesic ED50, and this indicated that the two effects were related. The relative analgesic and intestinal depressant activities of pethidine, dimethylthiambutene and phenadoxone were similar at all levels of response, and these compounds only affected intestinal transport at doses higher than those needed for a marked analgesic action. On the other hand, morphine and methadone delayed intestinal transport at doses lower than those causing appreciable analgesia in the rat.

In a second comparison of morphine, methadone, and pethidine, measuring analgesic and intestinal effects in the same dieted rats, the use of larger groups of animals and a greater dose range allowed more precise definitions of dose/response relationships. The slopes in the charcoal meal tests differed significantly from each other, being, for morphine, -32 ± 3.0; methadone, -43 ± 2.6; and pethidine, -89 ± 12.2. No differences were detected between the slopes in the analgesic tests. The mean and 95% limits of the doses in mg./kg. reducing propulsion by 50% were 1.22 (0.84 to 1.76), 0.95 (0.75 to 1.21), and 18.2 (14.7 to 22.4) for morphine sulphate, methadone hydrochloride, and pethidine hydrochloride respectively, and the corresponding estimates for each analgesic ED50 were 4.4 (3.2 to 6.2), 2.4 (1.4 to 3.9), and 14.0 (9.4 to 20) respectively.

The results of separate experiments on propoxyphene and codeine are included in Fig. 2. The regression for propoxyphene was linear, the slope being nearly as steep as that for pethidine. The values for codeine were not readily fitted by a straight line, and the dotted

TABLE II

THE EFFECTS OF SUBCUTANEOUS INJECTIONS OF ANALGESICS IN THE CHARCOAL MEAL TEST

The analgesic doses shown are those earlier found equivalent to 3 mg./kg. morphine sulphate in elevating the threshold to pressure: (a) Green and Young (1951); (b) Green (1953). Doses in mg./kg. 95% limits in parentheses.

Drug	Charcoal Meal Test			Equivalent Analgesic Doses
	Slope±s.e.	Dose for 50% Reduction	Dose for 100% Reduction	
Morphine sulphate	−41± 5·6	0·72 (0·50–1·04)	12	3·0
Methadone hydrochloride	−55± 9·7	0·79 (0·58–1·08)	6	1·3 (1·2–1·4) (a)
Pethidine ,, ...	−139±21·7	21·5 (19·0–24·4)	50	11·0 approx. (a)
Phenadoxone ,, ...	−189±43·2	0·73 (0·67–0·79)	1·5	0·60 (0·47–0·78) (a)
Dimethylthiambutene hydrochloride ..	−143±21·7	5·16 (4·30–6·21)	12	2·8 (2·4–3·3) (b)

line in Fig. 2 indicates that there seem to be two distinct components in its effect. Each drug largely prevented gastric emptying when about four times the analgesic ED50 was given, as has already been noted with the other compounds examined. Propoxyphene showed a constipating effect in the rabbit (Robbins, 1955).

Analgesics Given Intravenously.—Although the above tests did not discriminate between effects on the pylorus and effects on the propulsive mechanism of the small intestine, the latter were shown to play a major part in tests where the drugs were injected intravenously after a time which had been shown in control studies (Fig. 1) to be sufficient to allow charcoal to enter the small intestine. In an experiment using groups of five dieted rats, in which intravenous doses were given 2 min. after the charcoal, the mean percentage of the small intestine traversed in a total of 10 min. was 43 ± 5.4 (s.e.) in saline controls, 20 ± 7.1 after 2 mg./kg. morphine sulphate, 19 ± 8.2 after 2 mg./kg. methadone hydrochloride, 16 ± 6.1 after 20 mg./kg. pethidine hydrochloride, 7.6 ± 6.4 after 1 mg./kg. phenadoxone hydrochloride and 9.4 ± 8.6 after 5 mg./kg. dimethylthiambutene hydrochloride. The values for the groups given analgesics were not significantly different from one another, but all were significantly reduced below the control value. Though in this experiment the distance travelled by the charcoal at the time the analgesics were injected was not determined, in comparable tests the mean percentage travel in 17 rats killed 2 min. after giving the meal was 22 ± 4.0. Even though the control rate of propulsion was somewhat slower in this experiment than usual (10 min. travel 43%, compared with the overall control mean of 58%), it was clear that the intravenous doses of these analgesics, especially phenadoxone and dimethylthiambutene, must have abolished or nearly abolished propulsion. The intravenous doses were all between a quarter and a half of those which largely abolished propulsion in the subcutaneous tests (Fig. 2).

Peristaltic Reflex

Schaumann, Giovannini, and Jochum (1952) showed that the relative activities of morphine, pethidine, and the optical isomers of methadone in depressing the peristaltic reflex of isolated guinea-pig ileum were somewhat similar to their relative analgesic potencies in mice and rats. Several estimates of the log dilutions for causing slight and nearly complete inhibition of the reflex are summarized in Table III. Analysis of the tests on morphine, pethidine, methadone, and phenadoxone showed that the variance of the *p*D values was the same (0.04) for each drug at each level of effect, whether determined from the response of the longitudinal muscle or the volume change. Further, no difference was found

TABLE III

INHIBITION OF THE PERISTALTIC REFLEX OF ISOLATED GUINEA-PIG ILEUM

Means causing slight (±) and nearly complete (+++) inhibition of the reflex longitudinal muscle response and volume change; the potency ratios are derived from the mean of these *p*D values; n= number of observations on ileum from different guinea-pigs.

Compound	n	*p*D±	*p*D+++	Potency Ratio
Morphine sulphate	8	7·31	6·83	1·0
Codeine phosphate	3	6·53	5·20	0·063
Pethidine hydrochloride ..	6	6·44	5·84	0·12
Methadone hydrochloride ..	6	7·89	7·34	3·53
Phenadoxone hydrochloride ..	5	8·18	7·44	5·51
Dimethylthiambutene hydrochloride	2	7·45	6·75	1·07
Propoxyphene hydrochloride ..	3	6·75	5·83	0·16

TABLE IV

INTRACISTERNAL MORPHINE IN RATS

Effects at 30 min. after various doses in 0·05 ml. saline. Analgesia: nos. with squeak response thresholds and heat reaction times for tail flick response greater than twice control mean, and nos. with thresholds outside control 95% limits; respiratory frequency; % intestine traversed by a charcoal meal in 10 min.

	Control Mean±s.d.	Dose (mg./kg.)				
		0·005	0·02	0·08	0·32	1·28
No. in group	16	16	15	16	16	16
Pain threshold (cm. Hg) ..	8·9±2·4					
No. above 2 × control ..	—	1	4	13	16	16
,, 95% limit ..	—	1	7	15	16	16
Heat reaction time (sec.) ..	3·9±1·2					
No. above 2 × control ..	—	0	1	1	12	16
,, 95% limit ..	—	0	2	2	14	16
Respiratory frequency						
Mean/min.±s.d.	294±72	291±72	213±114	210±95	136±48	106±43
No. below 95% limit ..	—	0	5	7	11	14
% intestine traversed						
Mean ± s.d.	39±13	41±14	27±20	26±17	15±18	3±3
No. below 95% limit ..	—	0	4	4	10	16

between the *p*D values for the longitudinal muscle response and those for the volume change, and the differences between the values producing the two levels of effect were alike for the four drugs. The means of these two *p*D50 values were determined for each test and found to have an overall variance for all compounds of 0.027. The potency ratios in Table III were determined from the antilog of the difference between the mean of these values for each compound and that for morphine. These ratios are similar to the relative potencies determined in the intact rat, though two species and entirely different test conditions are concerned in such a comparison. The effects of all the analgesics on the peristaltic reflex were inhibited by nalorphine at 0.1 μg./ml.

The Effects of Morphine Injected Intracisternally

The effects in the rat of various doses of morphine injected intracisternally, on (*a*) the threshold pressure on the tail required to cause a squeak response, (*b*) the reaction time for a tail flick response when radiant heat was focused on the tail, (*c*) respiratory frequency, and (*d*) transport of a charcoal meal, are summarized in Table IV and Fig. 3. Several points of interest arise. First, all the effects occurred at much lower doses than with the subcutaneous route, for example, 50 to 100 times less for the pressure squeak response. Second, heat reaction times for the tail flick tended to be least affected, the ED50 being at least four times the ED50 for the pressure squeak response, whereas the difference was trivial by subcutaneous injection (Green and Young, 1951). This suggested that when given intracisternally morphine did not attain such a high concentration at the lower levels of the cord concerned with the tail flick reflex as it did in some parts of the brain. Third, the depression of respiration relative to the elevation of the squeak threshold tended to be greater after intracisternal than after subcutaneous injection; after intracisternal injection the concentration of morphine would be greatest near the medulla. Fourth, the effects on respiration and intestinal transport appeared at the same dose and increased roughly in parallel. The action of the intracisternal morphine on intestinal transport was so great as to indicate that a central action must be concerned in its effects on the alimentary tract when the drug is administered by other routes.

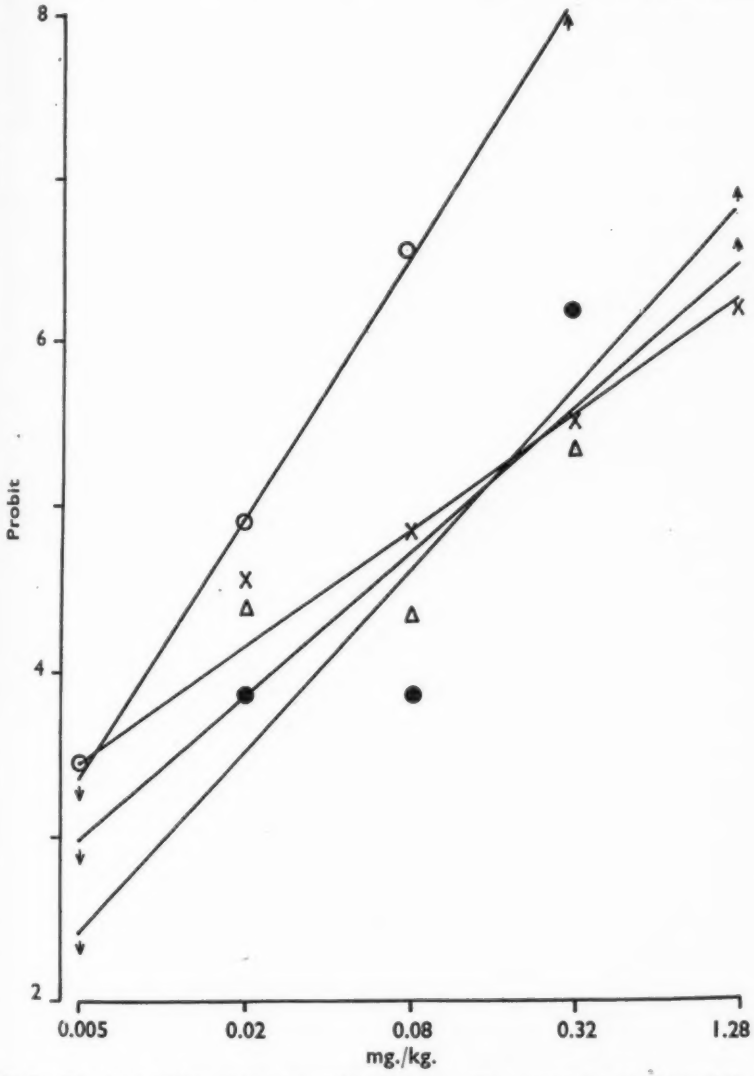

FIG. 3.—The effects of intracisternal morphine in rats. The regression on log dose of the probit of the number of animals in the group with values outside control 95% limits (see Table IV). O, pressure threshold for squeak response; ●, heat reaction time for a tail flick response; X, rate of breathing; Δ, % small intestine traversed by a charcoal meal.

Effects of Various Pharmacological Agents in the Charcoal Meal Test when Given Alone or in Combination with Morphine Sulphate

These effects are summarized in Table V and Fig. 4. Atropine reduced the passage of the charcoal but to a much smaller degree than morphine; its effect was synergistic with that of morphine. Pentobarbitone sodium also retarded propulsion but only in amounts that were near those producing anaesthesia; this substance and tribromoethyl alcohol greatly increased the effect of morphine even in amounts that when given alone had insignificant effects. Large doses of adrenaline delayed propulsion and were synergistic with morphine. No effect was produced by large doses of papaverine, dibenamine (50 mg./kg.), or the antihistamine *trans* - 1 - *p* - chlorophenyl - 1 - pyrid - 2′ - yl - 3 - pyrrolidin-1″-ylprop-1-ene hydrochloride (Green, 1953b) either alone or in combination with morphine.

Nalorphine reduced intestinal transport, though to a smaller extent than did morphine, and it antagonized the effect of morphine on propulsion (Fig. 4). The best antagonism of the effect of 10 mg./kg. morphine sulphate was seen with 10 mg./kg. nalorphine, the effect on propulsion then being less than with either compound alone; after 10 mg./kg. morphine and 30 mg./kg. nalorphine the effect was similar to that with large doses of nalorphine alone.

Discussion

The ratios of analgesic activity to respiratory depressant activity by the methods used here were the same for each of several analgesics representing diverse chemical groups. The only possible exception was pethidine, though even here the difference was not significant ($P=0.07$). An indirect comparison of the effects for morphine and the isomers of dextromoramide by de Jongh and van Proosdij-Hartzema (1957) revealed no dissociation of the analgesic and respiratory depressant properties, though in a later paper using the same results for respiratory depression but different estimates of analgesia obtained by another method, Janssen, Jagenueau, van Proosdij-Hartzema and de Jongh (1958) claimed that dextromoramide and a new pethidine analogue

TABLE V

CHARCOAL MEAL TEST: EFFECTS OF VARIOUS COMPOUNDS ALONE AND IN COMBINATION WITH MORPHINE OR METHADONE

Intestine traversed measured 10 min. after charcoal administration, 30 min. after the analgesics (s.c.) and at various intervals after treatment with the compound under test (s.c.). Means for groups of six rats and standard errors. Dose in mg./kg. unless stated. Morphine given as sulphate, 10 mg./kg., methadone as hydrochloride, 5 mg./kg. Bromethol was a 66·6% w/w solution of tribromoethyl alcohol in amylene hydrate diluted with saline.

Expt. No.	Compound	Dose	Interval (min.)	Mean % Intestine Traversed ± s.e. (or Range in Parentheses)	
				+ Saline	+ Morphine
1	Saline		15 and 30	64 ± 2·8	27 ±7·4
	Adrenaline tartrate	0·5 twice	15 and 30	32 ± 3·5	12 ±2·9
	Pentobarbitone sodium	20	30	26 ± 8·3	2·9±0·53
2	Saline		60	65 ± 4·1	12 ±2·8
	Atropine sulphate	0·25	60	39 ± 5·7	5·0 (0 to 26)
	,, ,,	1·0	60	32 ± 5·8	3·7±0·6
	,, ,,	4·0	60	27 ± 5·2	1·8 (0 to 5)
3	Saline		60	43 ± 3·5	20 ±3·2
	Atropine sulphate	5	60	34 ± 8·3	3·3±0·7
	,, ,,	50	60	22 ± 8·2	5·3±4·5
4	Saline		60	49 ± 4·1	12 ±3·2
	Papaverine HCl	10	60	54 ± 4·5	10 ±4·4
	,, ,,	100	60	56 ± 9·0	15 ±5·1
	Antihistamine 405C49	20	60	52±13·4	8 ±3·6
5	Saline		60	41 ± 4·1	16 ±5·0
	Pentobarbitone sodium	10	60	50 ± 3·6	5·3±1·6
	,, ,,	20	60	49 ± 2·4	9·8±6·1
	Bromethol	0·2 ml.	60	36 ± 4·9	4·2±2·1
	Dibenamine	50 mg.	60	44 ± 3·9	16 ±4·9
				+ Saline	+ Methadone
6	Saline		60	52 ± 4·1	0·83 (0 to 3)
	Atropine sulphate	1	60	23 ± 4·6	4·8 (0 to 11)
	,, ,,	10	60	27 ± 2·1	6·5±0·92
	,, ,,	100	60	6·3 (0 to 30)	1·1 (0 to 4)

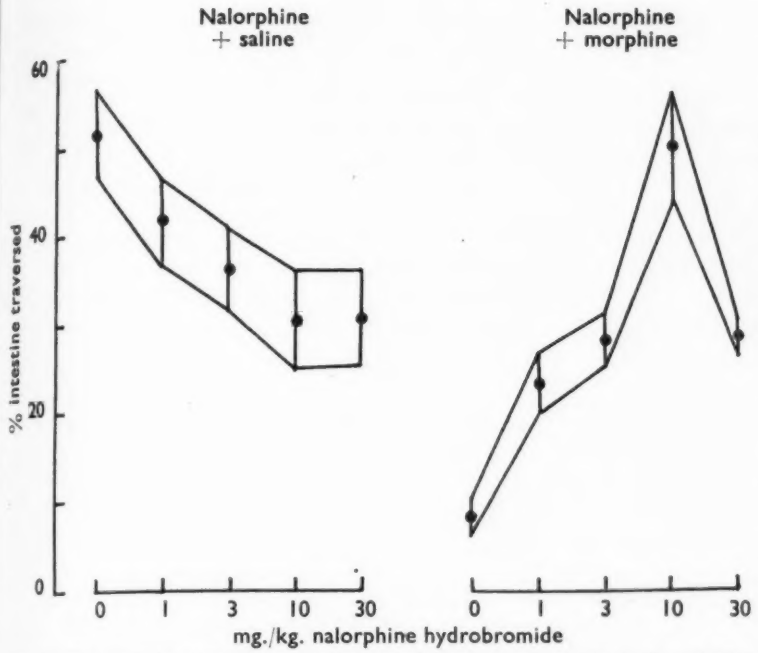

FIG. 4.—The percentage of small intestine traversed in 10 min. by a charcoal meal after nalorphine ± 10 mg./kg. morphine sulphate injected subcutaneously at the same time. Drugs given 20 min. before charcoal. Vertical lines represent s.e. (n = 10 for each point).

had relatively less effect than morphine on respiration. In our experiments, the respiratory depressant activity of dextromoramide was proportional to its analgesic activity. Our experience has been that estimates of effective doses may vary significantly from day to day in different batches of rats, and that only direct comparative tests are safe in studies of this kind. There are few comparable results published for other species. Interpretations have often been misleading, since the analgesic activity in one species (usually the rat) has not uncommonly been compared with respiratory depressant action in another (usually the rabbit). Such comparisons as have been made in the dog are limited by the small numbers of animals used. The analgesics which have not caused respiratory depression to the same extent as morphine in the dog would seem to be those with which a stimulant or convulsant effect supervened before high levels of analgesia are attained, as for example with (+)-methadone (Luduena and Ananenko, 1950) and propoxyphene (Robbins, 1955; Green, unpublished observations). In general the present results in the rat are in accord with those in man.

For each of several analgesics the amount that largely stopped the entry of a charcoal meal into the small intestine was about four times the analgesic ED50 value, and where the slopes of regression lines relating log dose to the proportion of the intestine traversed by the charcoal were parallel, the effect was proportional to the analgesic activity in different compounds. However, with morphine and to a lesser degree methadone, these slopes were shallow by comparison with those for pethidine, dimethylthiambutene and phenadoxone; in contrast with this group, morphine and methadone delayed transport of the charcoal at doses below those causing appreciable analgesia. A difference of this type between the effects of equiactive analgesic doses of morphine, methadone, and pethidine was reported by Karr (1947), but the relation of dose to effect was not studied. Tests of constipating effect in the rat, by counts of faecal pellets (see for example Schaumann *et al.*, 1952) are for comparative purposes open to the objection that since the counts are necessarily taken long after the main part of the analgesic action has passed off, the relative effects of compounds will depend as much on duration of action as on intensity of effect. A charcoal meal test has been used in mice by Janssen and Jageneau (1957) and Janssen *et al.* (1958), who gave analgesics 1 hr. before the charcoal and killed 2 hr. later to count the number of appendices blackened by the charcoal. Though this again may test the duration of action of the compounds rather than measure their activity, the various ratios of the ED50 obtained in this way to those for analgesic effects show a twenty-fold spread for different compounds, the distinction between morphine, methadone, and pethidine being small.

Vaughan Williams and Streeten (1950) made an extremely valuable study of the effects of morphine, methadone, and pethidine on fluid transport in cannulated Thiry-Vella loops in dogs. Since they did not make comparable observations on the analgesic potency of these compounds but relied on estimates reported for the rat, it is appropriate to compare their findings with those on analgesic activities in the dog obtained in this laboratory (Green, 1953a and unpublished observations). Their threshold subcutaneous doses for reducing fluid transport were 0.03, 0.08, and 1.7 mg./kg. for morphine, methadone, and

pethidine, respectively ; our estimates of the ED50 for elevating pain threshold by 100% in the dog are 1.0, 0.65, and 15 mg./kg. respectively. Hence for morphine, methadone, and pethidine the very approximate ratios of the equipotent doses on the bowel to the equipotent analgesic doses are 1:4:4 ; these ratios have wide limits, those of the analgesic comparisons alone being of the order of 60 to 180% of the means (Vaughan Williams and Streeten (1950) report the ratios of equipotent doses on the bowel of the dog to equipotent analgesic doses in the rat as 1:5:75, but from their observations the ratios are 1:5:7.5). The major difference between the results in the dog and those reported here for the rat is that only in the former were the regressions of effect on log dose the same for morphine, methadone, and pethidine. It may be significant, in relation to this difference, that the results for the dog involve only the small intestine, whereas in those for the rat an action on the pylorus may also be concerned. It has been shown by administration of analgesics intravenously that a major part of the delay in propulsion of the charcoal meal in the rat occurs in the small intestine, but the importance of an action on the pylorus has not been assessed. Two components seem to be concerned in the dose/response relationship of codeine, and it is possible that more detailed studies might reveal two components in the effects of other analgesics in this test.

Nalorphine was the only one of several pharmacological agents examined which antagonized the action of morphine in charcoal meal tests in rats. Atropine increased the effect of morphine in these tests, though Vaughan Williams and Streeten (1951) found that atropine antagonized the effect of moderate, but not large, doses of morphine on fluid transport in cannulated Thiry-Vella loops in conscious dogs.

The evidence concerning the possible sites of action of morphine on the alimentary tract has been reviewed by Krueger *et al.* (1941) and Reynolds and Randall (1957). The powerful inhibitory effects of several compounds on the peristaltic reflex of the isolated guinea-pig ileum described here, like similar findings of Schaumann *et al.* (1952) and Schaumann (1954 and 1955), emphasize the importance of a peripheral mechanism. The retardation of the propulsion of a charcoal meal caused by low intracisternal doses of morphine shows that a central site of action must be concerned in the rat. Similar findings were reported by Margolin (1954) for intracranial injections of morphine in the mouse, and mention was made of comparable effects in the rat and guinea-pig. Both central and peripheral sites are clearly of importance in the complex actions of morphine on the alimentary tract, but the importance of a central action in the corresponding effects of allied drugs has not been established.

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DISTRIBUTION OF [^{14}C]BEMEGRIDE IN TISSUES AFTER INTRAVENOUS INJECTION

BY

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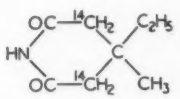
The distribution and excretion of bemegride labelled at the α carbon atoms with ^{14}C were studied in mice, rats, and guinea-pigs. These studies were supplemented by others made by colorimetric and absorptiometric methods on unlabelled bemegride in the body fluids of rabbits and dogs. Bemegride passed rapidly from plasma to all tissues, including brain, muscle, and fat. In addition it passed readily into the cerebrospinal fluid and the aqueous humour and to the foetus. It persisted in the tissues for longer than 24 hr., to some extent preferentially in the central nervous system. Approximately two-thirds of the dose was excreted in the bile, faeces, and urine within 24 hr. The glutarimide ring of bemegride was not completely degraded biologically.

Bemegride (β-ethyl-β-methylglutarimide) was introduced by Shaw, Simon, Cass, Shulman, Anstee and Nelson in 1954 as an antagonist of the effects of barbiturates. Conflicting statements have been made as to its specificity, effectiveness and toxicity when used for this purpose. Injected intravenously in animals or man in sufficient dosage (particularly as the soluble sodium derivative), it will induce convulsions, and in man non-convulsive doses may give rise to a period of restlessness and mental disturbance; if the animal or patient is in a comatose state induced by a barbiturate it may often bring about a rapid improvement in respiration, a rise in blood pressure and restoration of reflexes such as the righting reflex in rats and mice, or the withdrawal reflex on stimulating the sole of the foot in man. There is ample experimental evidence that "sleeping-time" and mortality from injected barbiturates in animals are much reduced by appropriate treatment with bemegride, but controversy persists as to whether or not the period of unconsciousness in man from a given barbiturate dosage is reduced. This doubt seems to be due largely to the failure by different authors to define the limits of consciousness and the type and blood level of barbiturate so that their work may be compared. The non-specificity of bemegride is no longer in doubt; it effectively rouses animals and patients narcotized by a variety of depressants of unrelated structure, but it has been effective in severe cases of barbiturate poisoning in man.

Relatively little seems to be known about the absorption and fate of this compound despite its use in therapeutics. Anderson (1958) described a spectrophotometric procedure capable of detecting $\beta\beta$-disubstituted glutarimides in biological fluids, and showed that bemegride disappeared rapidly from the blood stream after intravenous injection, 90% having gone within 20 min. This was attributed to a rapid and even distribution throughout the body and a slow rate of excretion in the urine. McCallum (1955) showed that bemegride was excreted by man partly unchanged and partly as β-(2-hydroxyethyl)-β-methyl-glutarimide.



A specimen (70 mg.) of bemegride labelled with ^{14}C at the two α positions was used to determine the distribution and fate of the drug. It had a specific activity of 8.5 μC./mg.

METHODS

The [^{14}C]bemegride was dissolved in saline to make a final concentration of 3 mg./ml. (25.5 μC./ml. activity).

Mice.—Groups of five male white mice of 20 to 25 g. wt. were injected intraperitoneally with pentobarbitone sodium 60 mg./kg. After 10 min. they were injected intravenously with 15 mg./kg.

[^{14}C]bemegride (approximately 2.5 μC./mouse). Each mouse was put at once into a glass chamber of 2 l. capacity through which 1.5 l. of air was pumped each minute. Food and water were provided after 4 hr. The outflow passed through a series of absorption tubes containing 10 N-NaOH. The trapped CO_2 was precipitated as barium carbonate, filtered, washed and suspended at a concentration of 100 mg./ml. for counting. The chamber was lined each time with fresh filter paper from which the urine was eluted with 50% aqueous methanol; any urine found in the bladder was added, and the whole dried, taken up in 2 ml. methanol/water and counted. The mice were removed after 5, 15 or 30 min. and 1, 2, 4, 8, 16 or 24 hr., decapitated and bled into a minimal volume of oxalate solution, 0.25 ml. from each being added to the pool from which samples were taken for counting. The carcasses were dissected immediately and the tissues rinsed in fresh saline to free them from blood, blotted dry, pooled by groups, weighed and stored at $-4°$ in plastic bottles. Brain, skin, fat, skeletal muscle, liver, lung, kidney, spleen, testis, thymus, small and large intestine wall, and washed out intestinal contents were collected. Soft tissues were ground with water to give a suspension containing 300 mg./ml. Muscle and scraped skin were hardened by immersion in liquid N_2, pulverized and ground up. Fat was extracted four times with 1 ml. 10% v/v methanol in chloroform with 30 min. shaking/extraction and the extract diluted with solvent to a concentration of 0.5 g. fat/ml. Free faeces were measured separately from the moist intestinal content, weighed, ground to a fine suspension of 300 mg. solid/ml. of water and counted.

Rats.—Four male hooded rats (210 to 240 g.) were fasted for 12 hr., dosed twice by stomach tube with 10 ml. warm water each at 1 hr. interval, injected intraperitoneally with pentobarbitone sodium 30 mg./kg. 30 min. later and the external jugular vein and the bladder cannulated. All urine was collected. 15 mg./kg. [^{14}C]bemegride (approximately 26 μC/rat) was injected intravenously 20 min. later and one rat killed at 15, 30, 60 or 120 min. Tissues and organs were dissected and prepared as for mice. Blood was separated into washed red cells, which were lysed in distilled water, and plasma. Protein was precipitated from the plasma by addition of 0.08 ml. of 50% trichloracetic acid/ml., centrifuged, washed three times and digested at 37° with 10 N-NaOH before counting.

Four female hooded rats in late pregnancy (230 to 270 g.) were similarly injected and killed after 15, 30, 60 or 120 min. Uterus, foetus, placenta and amniotic fluid were collected in addition to the tissues listed above.

Guinea-pigs.—Two males (240 to 360 g.) were anaesthetized with pentobarbitone sodium 60 mg./kg. intraperitoneally and the jugular vein and common bile duct were cannulated. After 20 min [^{14}C]-bemegride 7.5 mg./kg. (approximately 13 μC./animal) was injected intravenously and the bile collected at 15, 60, and 120 min.

Dogs.—Three male dogs were anaesthetized with pentobarbitone sodium 30 mg./kg. intravenously, the femoral vein cannulated and the cisterna magna punctured. A clear specimen of cerebrospinal fluid having been obtained as control, unlabelled bemegride sodium 20 mg./kg. was injected intravenously, and cerebrospinal fluid was again taken at 10 and 20 min. Bemegride was estimated by a modification of the colorimetric method of Sheppard, D'Asaro and Plummer (1956).

Rabbits.—Animals were anaesthetized with 25% urethane solution intravenously and a femoral artery cannulated. Bemegride sodium, 15 mg./kg., was injected intravenously, and cerebrospinal fluid, anterior chamber fluid and arterial blood were collected at 3, 5, 10 or 20 min. from three rabbits each. Bemegride was estimated by the method of Anderson (1958).

Radioactivity Estimations.—Scintillation counting using liquid phosphors was employed. The phosphor was 2:5-diphenyloxazole 7 g., *p*-di(5-phenyloxazol-2-yl)benzene 0.05 g., naphthalene 50 g., dissolved in 1:4-dioxan to 1 l., except for extract of fat, where the phosphor consisted of 4 g. *p*-terphenyl and 0.1 g. *p*-di(5-phenyloxazol-2-yl)benzene dissolved in 1 l. of toluene. The suspension (0.1 ml.) was pipetted into 5 ml. of phosphor solution in a glass dish, placed in the scintillator (Panax Universal USC/l), counting begun after 4 min. and approximately 10,000 counts taken. Phosphor decay due to visual light and count-rate decay due to settling of the suspension were minimal. All samples were counted in duplicate and recounted after adding an internal standard of 0.2 μC. [^{14}C]bemegride. The specific activity was expressed as μg. bemegride/g. tissue or as % total activity administered.

Results

Distribution of Bemegride

Following intravenous injection bemegride is cleared rapidly from the blood, so that after 5 min. only some 12% of the initial dose remains. The rate of loss decreases and at 2 hr. 6% of the initial dose is still present. After 8 hr. the amount in the blood is very low (some 0.01% of the total initial dose) but persists for 24 hr. or more. Fig. 1 compares the blood levels found by us in mice with those given by Anderson (1958) for guinea-pigs. They are similar.

The bulk of the bemegride in the blood is in solution. Only some 0.02% of the total in plasma is precipitated with the protein. Since this small amount remains constant for 2 hr., during which time some 94% of the initial total has left the blood, it may be assumed to be protein-bound in a stable form. Very little bemegride penetrates the red cell envelope. Radioactivity was recorded from haemolysed red

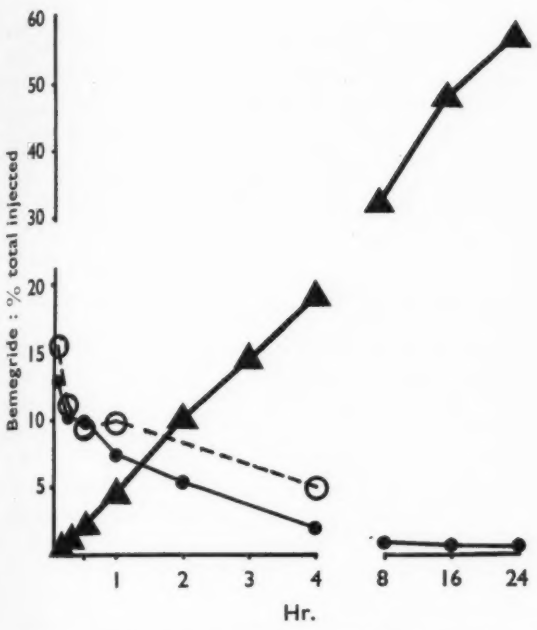


FIG. 1.—The concentration of [^{14}C]bemegride in blood (●) and urine (▲) of mice, expressed as % of the total intravenous injection (15 mg./kg.). Values for unlabelled bemegride in guinea-pig blood (O) are those given by Anderson (1958) for comparison. Note changes of scale in both axes.

TABLE I

DISTRIBUTION OF INJECTED [^{14}C]BEMEGRIDE IN RAT BLOOD

The concentrations at various times are expressed as μg. of bemegride/g. of tissue. Initial dose was 15 mg./kg. The amount in washed red cells or in the precipitated plasma protein (which may be bound and stable) remained constant over 2 hr. while the amount in whole plasma (probably free) fell steadily. The initial rapid fall was past before the estimates in this table were made.

Time (min.)	Plasma	Protein	Red Cells
15	21·7	0·87	0·29
30	19·2	0·89	0·56
60	18·7	0·96	0·30
120	13·0	0·89	0·28

cells, but as it only represented 0.007% of the total initial dose and as it remained more or less constant over the same period of 2 hr. the significance is doubtful; the activity might even be due to contamination of the cells by plasma or surface adsorption which resisted washing. These values for distribution within the blood after the initial rapid fall are shown in Table I, derived from the four male rats examined, expressed as μg./g. blood or blood fraction. In order to convert the values into percentages of initial total dose, the blood in the rat is taken as 7% of body weight.

The cause of the initial rapid fall in the concentration in the blood is shown in Table II, which gives the tissue concentration in mice at periods of time 5 min. to 24 hr. after intravenous injection. Bemegride passes rapidly into most tissues. Within a few minutes blood, brain, kidney, liver, spleen, and lung have similar concentrations, while those of the washed gut wall, fat, thymus, and testis are about 35 to 40% and skeletal muscle some 27% of that of the blood level. The skin value is low at 5%. This widespread distribution accounts for the rapid initial decrease in the concentration of bemegride in the blood. The ^{14}C activity is not found in the expired air and only increases in the urine at a steady rate. At 5 min. after injection into mice, when some 88% has left the blood only 0.06% is found in the urine. This increases steadily to 56% after 24 hr.

As with mice, this early and wide distribution is also found in rats of both sexes (see Table III); in addition it is evident that bemegride readily crosses the placental barrier and is found in the foetus in concentrations slightly above those in the maternal blood. It penetrates into amniotic fluid more slowly. The steadily increasing amounts in the intestinal content (upper small gut and whole gut washings in rats;

TABLE II

MEAN TISSUE CONCENTRATIONS IN GROUPS OF 5 MICE AFTER INTRAVENOUS INJECTION OF [^{14}C]BEMEGRIDE

15 mg./kg. of bemegride was injected and the concentrations of the drug at various times are expressed in μg./g. tissue or ml. blood. The urine and washings from gut lumen are expressed as % total dose. No $^{14}CO_2$ was detected in expired air at any time.

Time	Blood	Brain	Urine %	Kidney	Liver	Spleen	Lung	Gut	Gut Contents %	Muscle	Fat	Testis	Thymus
5 min.	27·3	23·9	0·06	25·3	33·7	23·5	17·4	9·8	0·5	7·2	11·7	11·3	10·6
15 ,,	22·7	14·9	0·8	30·8	36·1	52·9	38·7	13·8	1·1	7·4	10·6	11·5	10·3
30 ,,	19·5	12·5	1·8	29·6	26·8	44·7	25·2	13·1	1·9	6·9	10·5	9·7	8·7
1 hr.	15·8	10·0	4·4	28·5	30·3	51·4	36·0	11·9	1·4	9·8	7·1	10·0	13·8
2 ,,	11·8	8·3	10·2	22·9	22·4	31·8	23·6	11·4	2·2	7·9	7·7	11·6	17·3
4 ,,	4·2	4·7	18·8	22·6	15·4	34·7	15·1	4·4	0·7	2·7	2·5	5·3	8·6
8 ,,	1·5	4·6	30·7	6·7	6·9	2·6	13·7	2·7	0·4	1·1	0·6	2·3	4·6
16 ,,	0·2	2·7	46·8	4·8	4·6	2·9	8·2	0·7	0·4	0·3	0·5	0·7	2·8
24 ,,	0·2	2·8	56·1	5·9	6·1	8·0	4·1	1·0	0·4	0·4	0·5	0·8	2·9

TABLE III

TISSUE AND FLUID CONCENTRATIONS OF [^{14}C]BEMEGRIDE IN RATS WHEN INJECTED INTRAVENOUSLY

Concentrations are expressed in μg. bemegride/g. tissue or /ml. fluid. The urinary excretion at various times after the injection of 15 mg./kg. of bemegride is expressed as % initial dose. Each measurement was obtained from a single animal.

Time (min.)	Female Rats									Male Rats		
	Blood	Uterus	Placenta	Foetus	Amniotic Fluid	Liver	Kidney	Heart	Fat	Urine	Blood	Fat
15	20·5	6·4	16·5	24·6	7·5	26·9	19·6	14·6	5·2	0·3%	22·0	11·2
30	18·4	7·6	25·9	24·0	8·4	25·6	44·9	21·8	5·7	2·2%	19·8	12·9
60	17·1	10·0	21·2	24·1	9·2	27·8	29·7	24·3	5·0	2·7%	19·0	9·6
120	15·2	5·3	14·1	19·2	4·4	30·2	24·0	32·4	6·3	4·1%	13·3	6·7

total gut content of mice) are accounted for by the prompt excretion of bemegride in bile (see Table IV). In guinea-pigs, the quantity excreted by this route in 2 hr. amounts to 3.7% of that initially given. The biliary excretion (as measured from washings of the upper small intestine) would appear to be over more quickly in the rat than in the guinea-pig. The persistent difference between the concentrations from upper small intestine washings in male rats and the total gut content of female rats implies a sex difference in biliary excretion, or, more probably, excretion into the lumen in the initial phase when the blood concentrations are high, perhaps in digestive juices. The markedly lower concentration in fat than in blood found in mice is also found in rats.

Brain concentrations are similar to but somewhat lower than those in blood, implying that bemegride passes readily across the blood-brain barrier. This is supported by the evidence from rabbits shown in Table V. The arterial plasma concentrations in rabbits are similar to those in cisternal cerebrospinal fluid and in the aqueous humour in the anterior chamber of the eye (absorptiometric determinations). Calculation of the initial plasma concentrations in rats, mice, guinea-pigs, and rabbits from the expected blood volume and the known dose of bemegride indicate that the initial fall in concentrations of bemegride in blood is similar in all these species. The cerebrospinal fluid loses its bemegride somewhat more readily than does blood or anterior chamber fluid in the first half-hour, but brain as a whole retains it longer. The rapid early loss from cerebrospinal fluid may imply a passage into cells of the central nervous system.

The steady fall in the concentration of bemegride in blood and tissues and the rise in the urine continue after the initial rapid distribution. There is a tendency for the concentration in brain to decline more slowly than in other tissues. For example, at 8 hr. the blood concentrations decline to approximately 10% of those at 1 hr. and in liver, kidney, gut, and testis contents to approximately 20%, whereas the concentration in the brain only declines to 50%. Lung (approximately 40%) shows a similar tendency to retain bemegride, in contrast to the spleen (5%), another organ full of blood. There may be some reality, therefore, in the difference measured. After 24 hr. brain retains 28% of the concentration at 1 hr., kidney 21%, liver 20%, thymus 21%, spleen 15%, lung 11%, gut 8%.

TABLE IV

COMPARISONS OF RADIOACTIVITY IN BRAIN, BLOOD AND INTESTINAL CONTENTS OF MICE, RATS AND GUINEA-PIGS

The results are expressed as % of total intravenous dose of [^{14}C]bemegride, related to the weight of tissue or volume of fluid. The numerals in parentheses refer to the numbers of each species used.

Time (min.)	Mouse (5)	Male Rat (2)	Female Rat (2)	Guinea-pig (2)
	Whole Gut	*Proximal Gut*	*Whole Gut*	*Bile*
15	1·1	0·6	2·2	0·41
30	1·9	1·1	2·2	
60	1·4	1·0	2·7	2·1
120	2·2	0·3	2·4	3·7
	Brain	*Brain*	*Brain*	
15	2·1	1·2	1·6	
30		1·1	1·1	
60	1·1	0·9	0·9	
120	1·0	0·6	0·6	
	Blood		*Blood*	
15	10·9		9·6	
30	9·5		8·6	
60	7·6		8·0	
120	5·6		7·1	

TABLE V

CONCENTRATIONS OF UNLABELLED BEMEGRIDE IN ARTERIAL BLOOD PLASMA, CEREBROSPINAL FLUID AND AQUEOUS HUMOUR OF RABBITS AND IN CEREBROSPINAL FLUID OF DOGS

Both species were anaesthetized with urethane. Estimations, given in μg./ml., were made on rabbits using an absorptiometric method and in dogs with a colorimetric technique.

Time (min.)	Rabbit			Dog
	Plasma	C.S.F.	Aqueous Humour	C.S.F.
3	48·7	43·2	40·1	—
5	41·2	31·8	30·4	—
10	36·0	23·0	31·1	37
20	29·1	21·0	32·5	12

testis 8%, fat 7%, muscle 4%, and blood 1.3%. One may conclude that all the tissues retain bemegride for a long period of time (24 hr.) and in a variable concentration. The central nervous system does so preferentially to a moderate degree whereas fat does not. This trend becomes clear if we examine the brain/blood ratios at various times (Table VI).

TABLE VI

THE BRAIN/BLOOD CONCENTRATION RATIOS OF [^{14}C]BEMEGRIDE AT VARIOUS TIMES AFTER INTRAVENOUS INJECTION IN MICE

A low concentration of bemegride is retained in the central nervous system for a long period.

Time:	(Min.)			(Hr.)					
	5	15	30	1	2	4	8	16	24
Blood/brain ratios ..	0·92	0·70	0·60	0·69	0·70	1·3	3·3	15	15

Excretion

An important pathway of excretion is in the bile; excretion in the other digestive juices is probable. It is difficult to say how much is reabsorbed from the intestine, but a small proportion, perhaps 5% of the total, is finally lost in the faeces in 24 hr. The main pathway of excretion is in the urine. No product of a totally degraded molecule escapes from the lungs. The rate of urinary excretion is slow, the peak rate occurring at 8 to 16 hr. after injection. Only some two-thirds of the initial dose has been excreted in 24 hr. by these channels. The rest would appear to be retained in the tissues. Direct evidence of accumulation has not been produced in this study but may occur with a suitable dosage; a daily injection of 10 mg./kg. to growing rats for 30 days produces no detectable toxic effect.

DISCUSSION

The absence of radioactivity in expired air makes it clear that the ring structure of bemegride is not drastically fragmented by metabolic degradation. This deduction is supported by the findings of Anderson (1958) whose method of estimation of bemegride is dependent upon an intact glutarimide structure, and the finding by McCallum (1955) of a hydroxylation product in urine. The rapid and widespread distribution may well account for the speedy onset of the effects of bemegride which are in contrast to the delayed action of picrotoxin. The pK_a of 11.2 (Wiggins, personal communication) indicates that bemegride exists in the blood largely in an unionized form. It has a low coefficient of distribution between arachis oil and phosphate buffer (*p*H 7.4 at 37°) of 3.2, and is very soluble in fat solvents such as ether and chloroform. The content of bemegride in fat found after injection into the blood is consistently low and examination of residues has confirmed that this is a true state of affairs and not a misconception due to poor extraction from fat. Bemegride must, therefore, penetrate cells by some other route than the lipophilic one if such exists. The work of Kahn (1952) with picrotoxin and that of Achor, Geiling, and Domek (1956) with bemegride on the distribution of [^{35}S] thiopentone sodium implies that bemegride decreases the concentration of barbiturate in the central nervous system, reduces its storage in fat, and increases the rate of excretion in urine, while picrotoxin has no effect on distribution. These changes may have been due to the saline injected. No controls are mentioned. If this effect (essentially one on membrane permeability) is indeed the basis of the mode of action it must be effective at low concentrations of bemegride, because antagonism to barbiturate can be demonstrated in mice or men for 2 hr. or more after injection, and at that time the concentration in the brain and cerebrospinal fluid is very low. Also this effect must modify the distribution of other sedative-hypnotics in a similar way. It seems unlikely. The retention of one-third of the initial dose after 24 hr. suggests the possibility of a long persisting clinical activity and a low toxicity.

We are indebted to A. and G. Nicholas Limited for [^{11}C]bemegride. One of us (P. J. N) is in tenure of a Nicholas Scholarship of the Welsh National School of Medicine. We are indebted to Dr. F. H. Kendall, of the Sir John Cass College of Technology, for guidance and facilities in liquid phosphor scintillation work.

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Brit. J. Pharmacol. (1959), **14**, 40.

SOME EFFECTS OF RESERPINE AND HYDRALLAZINE UPON TISSUE RESPIRATION AND THE CONCENTRATION OF ADENOSINE NUCLEOTIDES IN CERTAIN TISSUES

BY

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The effects of reserpine and hydrallazine upon the oxygen uptake of preparations of rat brain, liver, and kidney and rabbit aorta have been investigated using the Warburg " Direct " method. In these tissues reserpine did not significantly stimulate respiration at any of the doses used. Concentrations of 10 or 50 μg./ml. had no significant effect upon respiration, but higher doses (330 μg./ml.) depressed it in all the tissues studied. This effect was also seen in rat brain at a reserpine concentration of 100 μg./ml. which had no significant effect in other tissues. Hydrallazine (50 or 100 μg./ml.) depressed respiration in all tissues studied. Stimulation at lower doses was not seen.

After sublethal doses of reserpine, depletion of energy rich phosphate compounds occurred in brain and liver but not in skeletal muscle and heart. Hydrallazine also depleted brain of energy rich phosphate compounds, but did not affect liver, heart, or skeletal muscle. The bearing of these results on the mode of action of reserpine and hydrallazine is discussed.

Earlier studies by Gillis and Lewis (1957) have indicated that reserpine may inhibit carbohydrate metabolism at some point in the tricarboxylic acid cycle and that it depressed respiration in slices of rabbit intestinal smooth muscle. We have shown that inhibition by hydrallazine of drug induced contractions of arterial smooth muscle can be antagonized by certain intermediates of carbohydrate metabolism and that the effects of hydrallazine on arterial strips resembled those of anoxia (Kirpekar and Lewis, 1958b). Reserpine was also found to inhibit drug induced contractions of arterial strips, but the effect was virtually irreversible and it was therefore not possible to study the effects of intermediary metabolites.

Abood and Romanchek (1957) have shown that in oxidative phosphorylation reserpine acts as an uncoupling agent and at a concentration of 2×10^{-5}M produced a fall of more than 40% in the P/O ratio of rat brain mitochondria. This value compares favourably with 2:4-dinitrophenol, a strong uncoupling agent. It is a characteristic of uncoupling agents that, at lower concentrations, they stimulate tissue respiration and at higher concentrations depress it. We have therefore investigated the influence of reserpine upon tissue respiration and compared its effects with those of hydrallazine. A compound which interferes with cell metabolism may influence the high energy phosphate level, especially that of adenosine triphosphate. We have therefore supplemented our studies on tissues respiration with estimations of the content of adenosine nucleotides in rat brain, heart, skeletal muscle, and liver, following treatment *in vivo* with reserpine and hydrallazine.

METHODS

For the determination of tissue respiration a 2.5 mg./ml. solution of reserpine was used which was diluted as required. The *p*H was 3.6. A control solution without reserpine was also used. An aqueous solution of hydrallazine HCl (20 mg./ml.) was prepared and diluted as required before use with Krebs-Ringer-phosphate solution (Umbreit, Burris, and Stauffer, 1957) (" phosphate medium ") or Krebs medium IIA (Krebs, 1950) (" medium IIA "). Addition of 0.4 ml. of reserpine or hydrallazine solution to the side arm gave a final flask concentration after tipping of 10, 50, 100, or 330 μg./ml. of the drug.

Respiration in Isolated Rat Tissues

Rats of either sex weighing from 150 to 200 g. were used. To obtain preparations of brain, the animals were killed, bled out, and the brain rapidly removed

and weighed. A 10% suspension was prepared by grinding the brain in a glass pestle and mortar with ice-cold phosphate medium. The suspension was filtered through muslin; 1 ml. of this extract (which is equivalent to 100 mg. of brain tissue) was used in each flask. Liver and kidney were removed from the animals, washed with phosphate medium to remove excess blood, and cut on a tissue slicer to give sections of 0.364 mm. thickness. A sufficient number of liver or kidney slices to weigh 100 mg. wet weight was used in each flask.

Respiration in Isolated Rabbit Thoracic Aorta

Rabbits weighing between 2.0 kg. and 2.5 kg. were killed by a blow on the head and bled out. About 5 cm. of thoracic aorta was rapidly removed and washed with ice-cold phosphate or IIA medium. The excised aorta was opened by a longitudinal incision and sliced to give pieces of the same thickness as liver and kidney. A sufficient number of slices to weigh 100 mg. wet weight was used in each flask.

Experiments on oxygen consumption were carried out by the Warburg "Direct" method at 37° using an atmosphere of air. The main chamber of each Warburg flask contained 2.4 ml. of medium together with the appropriate tissue preparation. The centre well contained 0.2 ml. of 20% KOH. The side arm contained 0.4 ml. of drug solution or the control, which in the case of reserpine was the solution supplied by the makers and of hydrallazine the appropriate physiological saline. After equilibration, the contents of the side arm were tipped into the main chamber and readings were taken at 10 min. intervals for 1 hr.

Estimation of Adenosine Nucleotides

Estimations were carried out by the specific enzymatic method of Kalckar (1947). Female albino rats weighing between 180 and 200 g. were used for these estimations. Rats were given 10 g. of powdered diet No. 86 (Laboratory Animals Bureau) for at least 5 days before killing. The powder was made into a paste with water to avoid spilling. Water was given *ad libitum*. This regimen was strictly adhered to since it has been shown by Munro and Goodlad (personal communication) that the adenosine triphosphate content of the liver is very sensitive to variations in dietary energy intake. Immediately after the last feed, 1 mg. of reserpine was given by intraperitoneal injection or 10 mg. of hydrallazine by subcutaneous injection. The controls were given equivalent volumes of the control solution or physiological saline respectively. Only those rats which had eaten all their food were used. Reserpine treated animals and their controls were killed between 11 and 12 hr. after injection; hydrallazine treated animals and their controls were killed 3 to 4 hr. after injection.

To minimize struggling, since such movements cause the breakdown of energy-rich phosphate compounds, the rats were injected with 200 mg./kg. of mephenesin 5 to 10 min. before killing. This usually gave complete muscular relaxation. Liver, heart, and gastrocnemius muscle were rapidly removed from the same animals, but brain was obtained from separate animals because of the length of time required for the dissection. Since doses of the drugs used were sublethal, estimations were always made after killing the animals. It was found, however, that 10 mg./rat of hydrallazine was quite toxic and some of the animals were in a poor condition while a few had convulsions and died. Animals which died due to drug administration were not used for experiments.

The animals were killed by cutting the throat. The tissues were removed and washed with ice-cold water; the excess water was removed by lightly touching with tissue paper, weighed on a torsion balance and ground up in 1.5% perchloric acid solution.

Skeletal Muscle. — Gastrocnemius muscle was ground up in a glass pestle and mortar. For each g. of muscle, 5 ml. of 1.5% perchloric acid solution at 0° was used. The contents of the mortar were transferred to a glass centrifuge tube and the mortar and pestle washed with two 5 ml. portions of 1.5% perchloric acid solution. The washings were added to the centrifuge tube and the contents centrifuged for 10 min. at 2,000 rev./min. at 0°. The supernatant was decanted into a measuring cylinder, and the residue was re-extracted with 5 ml. of 1.5% perchloric acid solution and centrifuged. The supernatant was then added to the stoppered measuring cylinder and the total volume noted. 10 ml. of the extract was pipetted into a beaker containing 10 ml. of succinate buffer at *p*H 6.1. The contents of the beaker were adjusted to *p*H 6.1 by addition of 5N-NaOH using the glass electrode. The volume was adjusted to 25 ml. with water, giving an approximately 1 in 50 tissue extract.

Liver.—About 2 g. of liver was excised and washed with ice-cold distilled water to remove excess blood; this was then transferred to 20 ml. of 1.5% ice-cold perchloric acid in a "Nalco" breaker vessel and ground. The suspension was transferred to a cooled centrifuge tube, the breaker vessel and the grinder blades rinsed with two 5 ml. aliquots of ice-cold 1.5% perchloric acid and the washings added to the suspension. The contents of the tube were centrifuged at 2,000 rev./min. at 0° for 10 min. and the supernatant transferred to a stoppered measuring cylinder. The residue was re-extracted with 5 ml. of 1.5% ice-cold perchloric acid, centrifuged, and the supernatant added to the measuring cylinder. The total volume was noted and a 10 ml. aliquot transferred to a beaker containing 10 ml. succinate buffer at *p*H 6.1. The *p*H was adjusted to 6.1 with 5N-NaOH and the final volume adjusted to 50 ml. to give an approximately 1 in 75 extract.

Heart.—Hearts were removed, washed with ice-cold water and extracted in a manner similar to that used for liver to give an approximately 1 in 75 tissue extract.

Brain.—Brain was extracted in a manner similar to that for liver and heart to give an approximately 1 in 50 tissue extract.

All tissue extracts were stored in the frozen state until assayed.

RESULTS

Tissue Respiration

Concentrations of 10, 50, or 100 μg./ml. of reserpine had no significant effects on tissue respiration except in rat brain when 100 μg./ml. caused depression ($P<0.01$). Reserpine (10, 50, or 100 μg./ml.) did not stimulate the oxygen uptake of kidney slices. Hydrallazine (50 or 100 μg./ml.) depressed the oxygen uptake of brain homogenates ($P<0.02$), liver slices ($P<0.01$), kidney slices ($P<0.01$), and arterial smooth muscle ($P<0.01$). Reserpine (330 μg./ml.) depressed the oxygen uptake of brain homogenates ($P<0.01$), and liver and kidney slices ($P<0.01$). The results are shown in Tables I and II. The control solution had little or no effect upon tissue respiration in brain but caused stimulation in liver ($P<0.01$). Previous work

TABLE I

EFFECTS OF RESERPINE AND HYDRALLAZINE ON THE OXYGEN UPTAKE OF BRAIN SUSPENSIONS, LIVER AND KIDNEY SLICES

Oxygen uptake is given in μl. ± s.e. mg. wet tissue. Control solutions: see text for details.

Tissue Solution	Conc. (μg./ml.)	No. of Observations	Incubation Time (min.)		
			20	40	60
Brain					
Reserpine	10	12	0·158 (±0·0089)	0·320 (±0·0099)	0·457 (±0·01)
Control		12	0·163 (±0·005)	0·316 (±0·005)	0·46 (±0·0075)
Reserpine	50	27	0·188 (±0·006)	0·328 (±0·0095)	0·476 (±0·0107)
Control		24	0·185 (±0 0063)	0·348 (±0·0102)	0·498 (±0·013)
Reserpine	100	13	0·162 (±0·0119)	0·292 (±0·012)	0·397 (±0·07)
Control		13	0·166 (±0·008)	0·318 (±0·0097)	0·477 (±0·007)
Reserpine	330	9	0·156 (±0·0085)	0·188 (±0·0106)	0·232 (±0·010)
Control		9	0·151 (±0·005)	0·282 (±0·011)	0·386 (±0·012)
Hydrallazine	50	11	0·122 (±0·011)	0·209 (±0·015)	0·268 (±0·018)
,,	100	8	0·154 (±0·025)	0·278 (±0·017)	0·323 (±0·010)
Control		30	0·179 (±0·008)	0·362 (±0·013)	0·514 (±0·016)
Liver					
Reserpine	10	8	0·347 (±0·030)	0·550 (±0·032)	0·764 (±0·033)
Control		7	0·294 (±0·0407)	0·516 (±0·029)	0·710 (±0·035)
Reserpine	50	18	0·434 (±0·0105)	0·7947 (±0·0148)	1·10 (±0·0146)
Control		12	0·404 (±0·0081)	0·7609 (±0·0151)	1·06 (±0·019)
Reserpine	100	10	0·4938 (±0·0116)	0·898 (±0·025)	1·222 (±0·0124)
Control		10	0·5200 (±0·0119)	0·935 (±0·020)	1·316 (±0·031)
Reserpine	330	8	0·3935 (±0·0031)	0·768 (±0·0106)	1·152 (±0·001)
Control		9	0·4659 (±0·011)	0·934 (±0·009)	1·378 (±0·0086)
Hydrallazine	50	14	0·151 (±0·014)	0·263 (±0·022)	0·478 (±0·02)
,,	100	11	0·111 (±0·003)	0·216 (±0·014)	0·311 (±0·016)
Control		41	0·201 (±0·007)	0·388 (±0·012)	0·658 (±0·013)
Kidney					
Reserpine	10	12	0·657 (±0·016)	1·276 (±0·021)	1·913 (±0·0245)
Control		12	0·6212 (±0·0181)	1·219 (±0·027)	1·817 (±0·036)
Reserpine	50	9	0·6627 (±0 036)	1·271 (±0 053)	1·90 (±0·089)
Control		10	0·608 (±0·026)	1·23 (±0·0359)	1·861 (±0·0616)
Reserpine	100	12	0·639 (±0·0275)	1·24 (±0·036)	1·837 (±0·047)
Control		11	0·648 (±0·0176)	1·272 (±0·0297)	1·883 (±0·0334)
Reserpine	330	9	0·484 (±0·0087)	0·821 (±0·041)	1·389 (±0·0356)
Control		9	0·536 (±0·013)	1·05 (±0·021)	1·589 (±0·0303)
Hydrallazine	50	6	0·389 (±0·035)	0·823 (±0·040)	1·20 (±0·04)
,,	100	6	0·541 (±0·045)	1·02 (±0·045)	1·46 (±0·05)
Control		12	0·63 (±0·016)	1·18 (±0·022)	1·75 (±0·04)

TABLE II

EFFECTS OF HYDRALLAZINE ON THE OXYGEN UPTAKE OF ARTERIAL SMOOTH MUSCLE

Oxygen uptake is given in μl. ± s.e./mg. wet tissue.

Solution	Conc. (μg./ml.)	No. of Observations	Incubation Time (min.)		
			20	40	60
Phosphate medium		11	0·12 (±0·013)	0·243 (±0·026)	0·373 (±0·019)
Drug	50	6	0·059 (±0·008)	0·125 (±0·013)	0·217 (±0·014)
,,	100	8	0·078 (±0·0027)	0·117 (±0·020)	0·170 (±0 032)
Medium IIA		11	0·173 (±0·019)	0·318 (±0·020)	0·414 (±0 028)
Drug	50	6	0·185 (±0·018)	0·335 (±0·028)	0·451 (±0·041)
,,	100	5	0·164 (±0·015)	0·230 (±0·015)	0·292 (±0·016)

(Kirpekar and Lewis, 1958b) had indicated that hydrallazine inhibition of drug induced contractions of isolated artery strips could be antagonized by certain intermediates of carbohydrate metabolism. We therefore carried out some experiments using medium IIA which contains pyruvate, glutamate, fumarate, and dextrose. In the presence of this medium, hydrallazine (50 to 100 μg./ml.) only slightly inhibited the respiration of slices of arterial smooth muscle.

Adenosine Nucleotides

Tables III and IV summarize the effects of reserpine and hydrallazine upon the amounts of adenosine nucleotides in rat skeletal muscle, heart, brain, and liver. Mephenesin administration does not affect tissue adenosine triphosphate concentrations (Parker, 1954). Mephenesin-induced relaxation was partially antagonized by hydrallazine, and rats treated with hydrallazine required one and a half to twice the usual relaxant dose of mephenesin, while the time of onset of relaxation was 20 to 25 min. as against the more usual 5 to 10 min.

Neither reserpine nor hydrallazine had a significant effect upon the adenosine nucleotide content of gastrocnemius muscle or heart. In the brain, reserpine caused a significant decrease in adenosine triphosphate (ATP) ($P<0.01$) and a corresponding increase in adenosine diphosphate (ADP) ($P<0.05$). The ATP/ADP ratio which may be taken as a measure of the synthetic potential of the cell is also significantly reduced ($P<0.01$). In liver reserpine caused a significant fall in adenosine triphosphate content ($P<0.01$) and there was a corresponding increase in adenosine diphosphate ($P<0.05$); the ATP/ADP ratio and the total adenosine nucleotides were also significantly reduced ($P<0.05$). Hydrallazine had no significant effect upon liver adenosine triphosphate: on the other hand, adenosine diphosphate was significantly increased ($P<0.05$) and the ATP/ADP ratio reduced ($P<0.01$). In brain, hydrallazine reduced the adenosine triphosphate level significantly ($P<0.01$), adenosine diphospate was significantly increased ($P<0.01$) and the ATP/ADP ratio was also significantly reduced ($P<0.05$).

TABLE III

EFFECTS OF RESERPINE AND HYDRALLAZINE ON ADENOSINE NUCLEOTIDES OF RAT SKELETAL MUSCLE AND HEART

All values are expressed in μmole/g. wet wt. ± s.e. ATP, ADP and AMP refer to adenosine tri-, di-, and mono-phosphate.

Group and No. of Rats	Solution and Dose (mg./100 g.)	ATP	ADP	AMP	ATP+ADP+AMP
Muscle					
1 (4)	Reserpine, 0·5	7·21 (±0·17)	0·35 (±0·19)	0·14 (±0·015)	7·52 (±0·36)
2 (4)	Hydrallazine, 5·0	6·88 (±0·33)	0·17 (±0·06)	0·05 (±0·02)	7·09 (±0·36)
3 (8)	Controls	7·57 (±0·31)	0·31 (±0·08)	0·12 (±0 05)	7·97 (±0·35)
Heart					
1 (4)	Reserpine, 0·5	3·27 (±0·35)	0·78 (±0·15)	0·60 (±0·12)	4·65 (±0·32)
2 (4)	Hydrallazine, 5·0	4·21 (±0·35)	0·79 (±0·15)	0·70 (±0·17)	5·71 (±0·17)
3 (8)	Controls	3·67 (±0·20)	1·18 (±0·17)	0·94 (±0·11)	5·79 (±0·23)

TABLE IV

EFFECTS OF RESERPINE AND HYDRALLAZINE ON ADENOSINE NUCLEOTIDES OF RAT BRAIN AND LIVER

All values are expressed in μmole/g. wet wt. ± s.e. ATP, ADP and AMP, see Table III.

Group and No. of Rats	Solution and Dose (mg./100 g.)	ATP	ADP	AMP	ATP + ADP + AMP	ATP/ADP
Liver						
1 (4)	Reserpine, 0·5	0·49 (±0·05)	1·16 (±0·07)	1·18 (±0 08)	2·82 (±0·19)	0·42 (±0 03)
2 (4)	Hydrallazine, 5·0	1·15 (±0·15)	1·47 (±0·19)	1·33 (±0·15)	3·95 (±0·18)	0·77 (±0 07)
3 (8)	Controls	0·91 (±0·03)	0·94 (±0·06)	1·48 (±0·09)	3·33 (±0·07)	1·00 (±0·061)
Brain						
1 (6)	Reserpine, 0·5	0·68 (±0·06)	0·50 (±0·08)	0·87 (±0·05)	2·02 (±0·10)	1·40 (±0·30)
2 (5)	Hydrallazine, 5·0	0·52 (±0 09)	0·55 (±0·09)	0·89 (±0·09)	1·96 (±0·07)	1·08 (±0·28)
3 (9)	Controls	1·10 (±0·06)	0·26 (±0·03)	0·90 (±0·07)	2·30 (±0·08)	4·74 (±0·88)

Discussion

Under normal conditions the oxidative metabolism of living cells and of certain isolated systems leads to the synthesis of compounds containing high energy phosphate bonds. These compounds are essential for growth, muscle contraction, nerve conduction, etc. Uncoupling agents can disturb metabolism by depressing the formation of high energy phosphate bonds without depressing or even slightly stimulating the oxygen consumption of the system (Brody, 1955).

Reserpine depresses the P/O ratio (Abood and Romanchek, 1957) in brain mitochondria—a property it shares with other uncoupling agents. On the other hand, hydrallazine depresses tissue respiration at all concentrations used, thereby indicating a general depressant effect upon the oxidative metabolism of living cells. Hydrallazine therefore appears to act differently from reserpine, which at lower doses (10 μg./ml. and 50 μg./ml.) has no significant depressant or stimulant effects.

Douglass, Dillaha, Dillaha, Janis, and Kountz (1957) have shown that hydrallazine inhibits the acetylation of sulphanilamide and glucosamine in pigeon liver extracts and have concluded that this inhibition is of a competitive nature and appears to be the result of the drug being preferentially acetylated by acetyl-coenzyme A. The acetylation of hydrallazine by acetyl - coenzyme A may ultimately mean interference in the functioning of the tricarboxylic acid cycle. Our previous finding (Kirpekar and Lewis, 1958b) that some intermediates of the tricarboxylic acid cycle antagonize the effects of hydrallazine supports this view. Our observation, in experiments on the respiration of arterial smooth muscle, that the inhibition of oxidation by hydrallazine can be overcome to a certain extent by supplying the tissue with pyruvate, glutamate, fumarate, and dextrose, also points in the same direction.

The effects of reserpine on the brain and liver adenosine nucleotides suggest interference with oxidative phosphorylation. No effect could be demonstrated in skeletal muscle or heart. It is possible that in these tissues phosphocreatine levels are affected. The accepted role of phosphocreatine is to maintain the level of adenosine triphosphate through the Lohmann reaction. Therefore, in the absence of the major synthetic source of energy-rich phosphate bonds, the loss of phosphocreatine will be the first indication of a disturbed phosphate metabolism. We are investigating this. Hydrallazine causes a decrease in the adenosine triphosphate level of the brain which is accompanied by an increase in the adenosine diphosphate level. This suggests that at any rate, in brain *in vivo*, hydrallazine also interferes with normal oxidative processes. This drug, however, had no effect on the adenosine nucleotide content in skeletal muscle, heart, or liver.

Many substances which uncouple phosphorylation from oxidation have been employed in experimental pharmacology and therapeutics. A number of antipsychotic drugs and central nervous system depressants inhibit oxidative phosphorylation: chlorpromazine and barbiturates are examples. Whether the effectiveness of these substances as drugs is dependent upon their effectiveness as uncoupling agents is not yet clear. The fact that a substance uncouples phosphorylation from oxidation does not mean that it is devoid of other actions. The relative importance of uncoupling as a mechanism of drug action differs with different drugs. Brody (1955) concludes that uncoupling is of major importance in the action of certain drugs and is at present the most attractive hypothesis, which explains, at least in part, the mechanism of action of barbiturates, antibiotics (such as gramicidin and the tetracyclines), salicylates, and thyroid hormones.

Reserpine has been shown to cause depletion of noradrenaline and 5-hydroxytryptamine from brain and other tissues (Holzbauer and Vogt, 1956; Pletscher, Shore, and Brodie, 1955). Carlsson and Hillarp (1956) have shown that the depletion of the adrenal medulla of catechol amines by morphine or insulin is accompanied by a parallel depletion of adenosine triphosphate. Depletion of this substance in rat brain may be linked with depletion of brain catechol amines. The observations of Kirpekar, Goodlad, and Lewis (1959) have recently shown that reserpine depletes adrenal medullary catechol amines and adenosine nucleotides in roughly the same proportions. There appears, however, to be a fundamental difference between the effects of reserpine on the adrenal medulla and brain since, in the former, depletion of adenosine triphosphate is not accompanied by a corresponding rise in the di- and mono-phosphates, and in the latter the fall in the triphosphate concentration is accompanied by a corresponding and expected rise in that of the diphosphate. In the case of hydrallazine, *in vitro* studies (Kirpekar and Lewis, 1957, 1958a, 1958b) have indicated a mainly peripheral site of action and have led us to

conclude that this drug is a general depressant of cell metabolism. The effect of this compound on brain adenosine nucleotide level was therefore not unexpected: but we cannot yet explain the lack of effect on skeletal muscle, heart, and liver.

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THE EFFECT OF RESERPINE ON THE ACETYLCHOLINE CONTENT OF DIFFERENT AREAS OF THE CENTRAL NERVOUS SYSTEM OF THE DOG

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The acetylcholine content of the frontal and temporal lobes, hippocampus, hypothalamus, cerebellum and spinal cord was estimated in 18 dogs 3 hr. after the intravenous administration of reserpine. Another group of 7 dogs served as control. After reserpine (0.5 mg./kg. body weight) there was an increase of acetylcholine in all the areas under study except in the hippocampus. The increase was specially marked in the hypothalamus. In the hippocampus, however, reserpine caused a significant decrease of acetylcholine.

In spite of the large amount of experimental work done with reserpine, the mechanism of its action still remains obscure. Anand, Dua, and Malhotra (1957) showed by implanting electrodes in the hypothalamus that reserpine not only depressed the sympathetic centres in the diencephalon, but also facilitated the parasympathetic centres. Holzbauer and Vogt (1956) found that the noradrenaline concentration in the hypothalamus of the cat was reduced by a single injection of reserpine. Pletscher, Shore, and Brodie (1956) observed that reserpine caused a severe loss of 5-hydroxytryptamine from brain tissue, especially from the hypothalamus. Brodie and Shore (1957) attributed the action of reserpine to the release of 5-hydroxytryptamine in the free form from depots in the brain and also considered it to be a possible central parasympathetic transmitter. It was considered worth while to study the effect of reserpine on the acetylcholine content of different areas of central nervous system as no work had been done on this subject and because acetylcholine has already been established beyond doubt to be the chemical transmitter of nerve impulses at some sites in the peripheral autonomic nervous system.

METHODS

Twenty-five dogs were used. Seven served as controls and the remaining eighteen received 0.5 mg./kg. body weight of reserpine intravenously. The animals were anaesthetized with ether, the skull opened and the whole brain removed as quickly as possible 3 hr. after the dog had received reserpine. It was immediately transferred to a beaker which had been kept in a freezing mixture. With the beaker still in the mixture the following portions of the brain were removed with scissors and placed in labelled weighing bottles that had also been kept in freezing mixture: (a) The anterior portion of the frontal lobe, (b) part of the temporal lobe including the periamygdaloid region, (c) part of the hippocampus, (d) the entire hypothalamus, (e) part of the cerebellar grey matter, and (f) part of the cervical segments of the spinal cord.

Acetylcholine was extracted from these portions of the central nervous system and assayed by the method of Nachmansohn described elsewhere (Anand, 1952). A control experiment was usually placed between two or three experiments with reserpine. Estimates of acetylcholine in the control animals were compared with those obtained treated with reserpine.

RESULTS

The estimates of acetylcholine in control dogs and dogs which had received reserpine are given in Table I. It will be seen that acetylcholine was widely though unevenly distributed in the central nervous system of the control group, the amount being highest in the hypothalamus and lowest in the cerebellum. The areas comprising the limbic system, the so-called " visceral brain," namely the hippocampus, that portion of the temporal lobe which includes the periamygdaloid region and the anterior portion of the frontal lobe, contained appreciable amounts of acetylcholine though less than the hypothalamus. On comparing the average values of acetylcholine in the dogs which received reserpine with those obtained from controls, it is clear that the administration of reserpine resulted in a significant increase in the

TABLE I

ACETYLCHOLINE CONTENT OF DIFFERENT AREAS OF DOG CENTRAL NERVOUS SYSTEM

Results are expressed in μg./g. tissue. Probability of differences between means of treated and untreated animals being due to chance also shown at the foot of the table.

Dog No.	Hypo-thalamus	Hippo-campus	Tempo-ral Lobe	Frontal Lobe	Cere-bellum	Spinal Cord
Control						
1	2·80	2·65	2·20	2·13	1·05	1·30
2	3·70	2·47	2·63	2·32	1·09	1·07
3	3·60	3·60	2·57	1·38	1·04	1·75
4	3·35	3·03	2·82	1·22	0·93	1·15
5	2·86	3·25	2·17	1·40	1·00	1·47
6	3·69	2·85	2·05	1·50	0·88	1·73
7	3·75	3·60	2·00	1·90	1·02	1·24
Mean	3·39	3·06	2·43	1·69	1·00	1·39
± s.d.	±0·406	±0·443	±0·307	±0·422	±0·025	±0·271
Treated						
1	3·71	2·52	2·17	2·14	1·27	1·72
2	3·76	2·38	2·95	2·46	1·35	1·55
3	4·27	1·98	2·94	3·21	0·95	1·45
4	4·39	2·17	2·82	1·76	1·20	1·72
5	5·56	2·94	3·05	4·27	1·10	1·58
6	5·72	4·62	3·52	3·80	2·35	3·05
7	3·60	1·72	2·62	1·93	1·05	1·57
8	2·99	1·28	2·71	1·75	1·25	1·95
9	3·67	1·80	2·92	2·52	1·44	1·72
10	2·98	1·98	2·06	1·56	1·15	1·51
11	5·45	2·60	3·80	2·54	1·28	1·59
12	4·58	1·96	2·55	1·98	1·45	1·95
13	3·80	2·25	2·77	2·25	1·04	1·75
14	3·45	1·85	2·22	2·00	1·06	1·84
15	5·56	2·44	3·22	1·87	1·37	1·43
16	4·43	1·98	2·85	2·28	1·15	1·56
17	4·55	2·13	3·15	2·22	1·27	1·75
18	3·99	1·42	2·90	1·99	1·39	1·58
Mean	4·25	2·22	2·85	2·36	1·28	1·74
± s.d.	±0·866	±0·725	±0·440	±0·718	±0·302	±0·361
P	<0·02	<0·01	<0·05	<0·05	<0·05	<0·05

acetylcholine content of all areas of the brain under study except hippocampus, the acetylcholine content of which actually decreased.

The observation that reserpine caused a decrease in the acetylcholine content of the hippocampus is quite significant and interesting. In this connexion, Anand, Malhotra, Singh, Pundlik, and Chhina (1958) found that although hypothermia caused a decrease in the acetylcholine content of the hypothalamus, the frontal and temporal lobes of the brain and of the heart, it increased the acetylcholine content of the hippocampus. This area of the brain seems to be affected in a direction opposite from that of the other areas as far as its metabolic pattern is concerned. The morphological appearance and the biochemical constitution of the hippocampus has been investigated by Lowry, Roberts, Leiner, Wo, Fan, and Albens (1954). It has been pointed out that it differs from other regions of the cortical surface, such as the frontal and occipital lobes, in several respects.

Kim and Maclean (1956), while comparing the effects of reserpine on the electrical responses of the limbic and neocortical structures, found that about 4 hr. after the administration of reserpine the recordings from the hippocampus began to show much rhythmic activity at a frequency of 3.5 to 4 cycles/sec. This activity was conspicuous only in the waking state during which the neocortex showed desynchronization. The contrast between the electrical activities of the hippocampus and neocortex becomes even more marked 20 hr. after giving reserpine. The available evidence therefore suggests an important inverse relationship between the hippocampus and other areas of the brain as far as their metabolic patterns are concerned, and the hippocampus may play an important role in autonomic function.

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SOME QUANTITATIVE USES OF DRUG ANTAGONISTS

BY

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Various applications of pA_x measurements are discussed based on the hypothesis that drugs and drug antagonists compete for receptors according to the mass law. Examples are given illustrating the use of pA_x measurements to identify agonists which act on the same receptors and to compare the receptors of different tissues. Tests of competitive and noncompetitive antagonism are considered in relation to the antagonisms acetylcholine-atropine, histamine-atropine and acetylcholine-cinchonidine. A new measure, pA_h, is introduced to express the activity of unsurmountable antagonists.

Although drug receptors have not so far been identified by physical or chemical methods, they can be identified pharmacologically by means of antagonists. If two agonists act on the same receptors they can be expected to be antagonized by the same antagonist, and, if the antagonism is competitive, they can be expected to be antagonized by the same concentration of antagonist and to produce with it the same pA_x (Schild, 1947a) or dose ratio (Gaddum, Hameed, Hathway, and Stephens, 1955); pA_x values can thus be used to identify agonists which act on the same receptors. They can also be used to identify receptors in different tissues since tissues with similar receptors would be expected to give the same pA_x with antagonists. Lastly, pA_x measurements can be used to distinguish between competitive and noncompetitive antagonists.

In this paper, examples of various applications of pA_x are given and the underlying assumptions and theoretical implications are discussed. Methods of measuring the activity of "unsurmountable" antagonists (Gaddum *et al.*, 1955) are also considered. Some of these results have already been referred to in a review article (Schild, 1957).

METHODS

In experiments on isolated tissues, automatic assay apparatus (Boura, Mongar, and Schild, 1954) was used. Drugs were administered either by automatic syringes, or by total displacement of the bath fluid by the drug solution. Unless otherwise indicated, pA_x values were determined by the method of Schild (1947a).

For the experiments on guinea-pig lung, an air perfused preparation was used modified from that of Sollman and v. Oettingen (1928) but differing from it in that air instead of fluid was perfused through the bronchial tree (see also Arunlakshana and Schild, 1950). The air perfusion system, illustrated in Fig. 1, consisted of a constant pressure head and two consecutive air flow resistances between which the pressure was measured. The first of these resistances consisted of a length of thermometer tubing and the second of the variable resistance of the bronchial tree from which air was allowed to emerge through scarifications on the surface of the lung. The air pressure was recorded by a mercury manometer the excursions of which are amplified by a frontal writing lever. Connection between manometer and lever was provided by a thread attached to a brass-weighted ebonite float resting on the surface of the mercury. The pull of the lever was adjusted to keep the thread taut without lifting the float from the mercury; in this way small excursions of the mercury manometer are amplified 5 to 10 times. An air flow of about 1 cm./min. was maintained with a pressure head of 100 cm. of water.

Guinea-pigs of 600 to 700 g. weight were used. The lungs were removed and immediately perfused with Tyrode solution at 37° through the pulmonary artery. The trachea was then joined to the air perfusion system and the surface of the lung gently scarified with the point of a fine needle. The preparation was placed in a water-jacketed moist chamber at 37°, and the protruding parts of the trachea were painted with liquid paraffin to prevent drying.

In newly set up preparations, the bronchial pressure record usually exhibited intermittent fluctuations presumably related to the rhythmic contractions of bronchi described by Einthoven (1892). The spontaneous movements generally subsided in the course of the experiment. Injections

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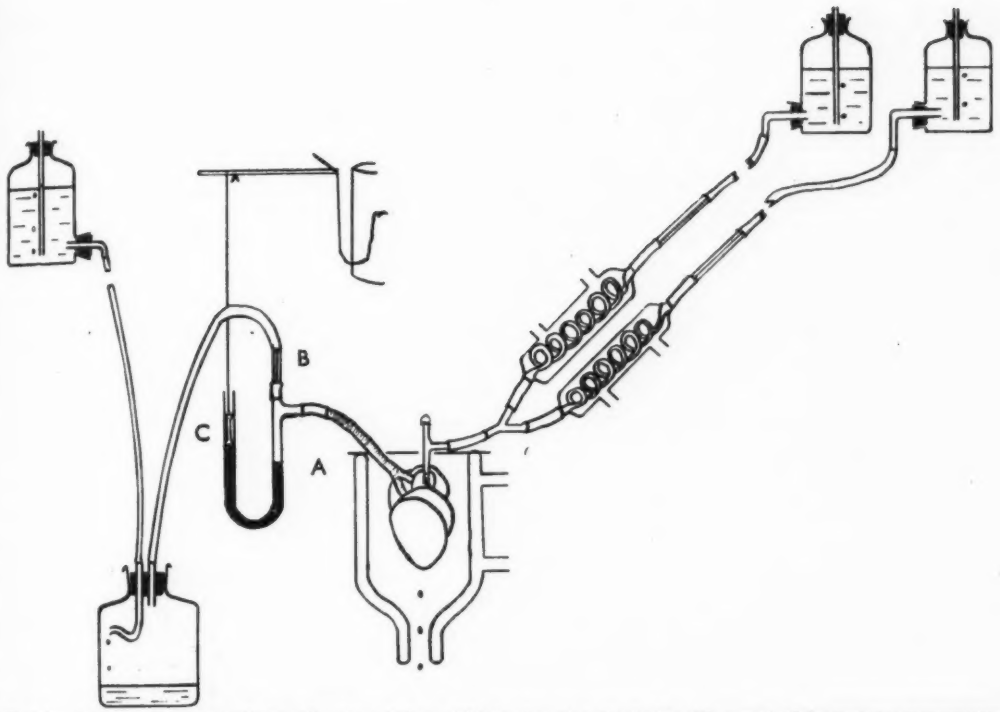

FIG. 1.—Apparatus for air perfusion of guinea-pig lung. The trachea was attached to side-arm A of the mercury manometer. Air at constant pressure passed through capillary resistance B and emerged through scarifications on surface of lung. C=weighted ebonite float attached to frontal writing lever. Prewarmed Tyrode solution flowed into pulmonary artery.

into the perfusion fluid of single doses of histamine and acetylcholine produced rapid and reversible rises of bronchial pressure lasting about 10 min. An approximately linear log dose/response relation was obtained over a fourfold range when the area of the time/response curve was used as the response metameter (Fig. 2). When drugs were administered at 15 min. intervals, the responses tended to increase for the first 3 to 4 administrations and then became reasonably stable.

β-2-Pyridylethylamine was obtained through the courtesy of the Maltbie Chemical Co. The other drugs used are standard commercial preparations.

THEORETICAL CONSIDERATIONS

Tests of Competitive and Noncompetitive Antagonism

The mass law equations as applied to drug antagonism refer to events on receptors rather than to observable responses, and in applying these equations it is necessary to postulate some relation between receptor activation and response. It has sometimes been assumed that the response is a linear function of the number of activated receptors, but more usually the more limited assumption has been made that equal effects in the absence and presence of antagonist involve equal numbers of receptors. This limited assumption, which does not specify the relation between receptors and response, underlies the use of pA_x (and the dose ratio) in testing for competitive and noncompetitive antagonism.

Use of pA_x to Test for Competitive Antagonism

Consider the competitive equation

$$y=\frac{K_1\ A}{K_1\ A+1}=\frac{K_1\ x\ A}{K_1\ x\ A+K_2B^n+1}$$

where y is the fraction of activated receptors, A and B are concentrations of agonist and antagonist respectively, x is the dose ratio, and K_1, K_2, and n are constants. Since by definition $pA_x=-\log B$ (Schild, 1949)

$$\log\ (x-1)=\log\ K_2-n\ pA_x \qquad (1)$$

Thus when $\log\ (x-1)$ is plotted against pA_x a straight line results with slope $(-n)$. This line intersects the pA_x axis at a point corresponding to pA_2. When $n=1$

$$\log K_2=pA_2$$

$$\text{and } pA_2-pA_{10}=0.95$$

The pA_2-pA_{10} difference can thus be used to test for a simple competitive antagonism, but for technical reasons, to be discussed later, it is

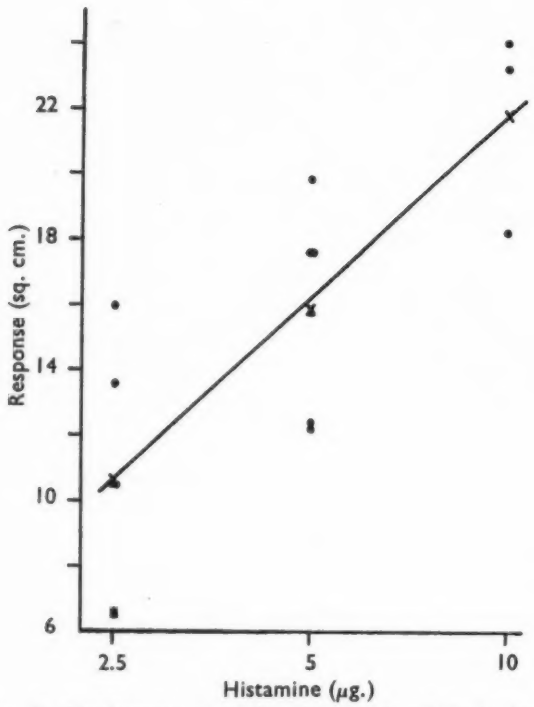

FIG. 2.—Dose/response curve for air-perfused guinea-pig lung. Ordinate represents area of curve above base-line following administration of histamine.

better to employ a wider range of concentrations of antagonist and test for competitive antagonism by means of equation (1). It should be noted that both tests are independent of y and thus require no knowledge of the fraction of receptors activated.

Competitive Drug Antagonism and Parallel Log Dose / Effect Curves

It can be shown that competitive antagonists produce parallel log dose/effect curves and that parallel log dose/effect curves can be fitted by equations of competitive antagonism.

(1) Consider first a simple competitive antagonism. If two responses correspond to activated receptor fractions y_1 and y_2

$$\frac{y_1}{1-y_1}=K_1\ A'=\frac{K_1\ A'\ x_1}{K_2\ B+1}$$

$$\frac{y_2}{1-y_2}=K_1\ A''=\frac{K_1\ A''\ x_2}{K_2\ B+1}$$

$$\therefore\ K_2\ B+1=x_1=x_2$$

$$\therefore\ \log\ x_1\ =\log\ x_2$$

Hence y_1 and y_2 are equally displaced when plotted on a logarithmic dose scale, and consequently the dose/response curves without and with antagonist are parallel.

(2) Consider next a competitive antagonism with two affinity constants for the antagonist. The equilibrium equations are

$$K_1=\frac{y}{x\ A\ (1-y-z_1-z_2)}$$

$$K_{21}=\frac{z_1}{B^m\ (1-y-z_1-z_2)}$$

$$K_{22}=\frac{z_2}{B^n\ (1-y-z_1-z_2)}$$

where z_1 and z_2 are the fractions of receptors occupied by antagonists corresponding to affinity constants K_{21} and K_{22}, m and n are the number of molecules of antagonist per receptor, and the other terms retain their previous significance. Eliminating z_1 and z_2

$$\frac{y}{1-y}=\frac{K_1\ A\ x}{K_{21}\ B^m+K_{22}\ B^n+1}$$

In the case of more than two affinity constants for the antagonist the following general equation obtains:

$$\frac{y}{1-y}=K_1\ A=\frac{K_1\ x\ A}{1+K_{21}\ B^m+K_{22}\ B^n+\quad\ldots\ldots}$$

$$\therefore\quad x=1+K_{21}\ B^m+K_{22}\ B^n+\quad\ldots\ldots$$

The same argument can now be applied as in section (1) above to prove that the log dose/effect curves with and without antagonist are parallel.

(3) To prove the converse, namely that parallel log dose/effect curves can generally be fitted by an equation of competitive antagonism, it is necessary to show that a general function $x=f(B)$ exists linking any horizontal displacement (log x) with concentration (B) of antagonist. But such a function must exist since x is a polynomial function of B and it is always possible to fit by means of a polynomial a series of experimental points.

To summarize, it is generally possible to fit an equation of competitive antagonism to a set of parallel log dose/effect curves although the equation may be complicated and involve several affinity constants. On the other hand, it is obvious that parallel log dose/effect curves are no proof of a competitive antagonism, indeed they may occur in neutralization antagonism as shown by Gaddum (1943).

The Use of pA_x to Test for Noncompetitive Antagonism

Consider the equation of noncompetitive antagonism (Schild, 1954):

$$y=\frac{K_1\ A}{K_1\ A+1}=\frac{K_1\ x\ A}{K_1\ x\ A+1}\ \ \frac{1}{K'_2B+1}$$

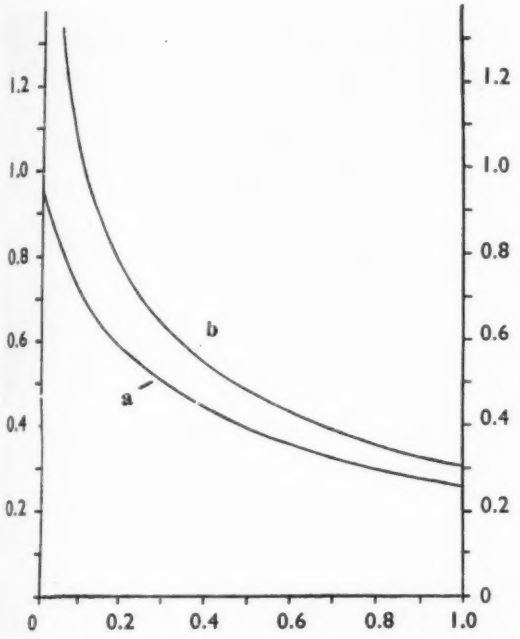

FIG. 3.—Abscissa: fraction of receptors occupied by agonist. Ordinate: theoretical values of (*a*) pA_2-pA_{10}, (*b*) pA_2-pA_h for a noncompetitive antagonist.

In this type of antagonism the log dose/effect curves are not parallel but become progressively flatter and their maxima decline. Both the pA_x values and the pA_x differences now depend on y. The relation between (pA_2-pA_{10}) and y in this type of antagonism is given by curve **a** in Fig. 3; it is seen that when $y=0.5$, $pA_2-pA_{10}=0.39$, but when $y\to 0$, $pA_2-pA_{10}\to 0.95$ as in competitive antagonism. For the calculation of K'_2 from the above equation it is necessary to know the value of y, but when y is unknown K'_2 may nevertheless be calculated from the following expression:

$$K'_2=\frac{x_1\ x_2\ (B_2-B_1)+x_1\ B_1-x_2\ B_2}{B_1\ B_2\ (x_2-x_1)} \quad (2)$$

where x_1 and x_2 are the drug ratios corresponding to antagonist concentrations B_1 and B_2.

The Use of pA_h

One of us (Schild, 1957) has proposed that the activity of antagonists which reduce the attainable maximum of the dose/response curve be expressed in terms of pA_h, which is defined as the negative logarithm of the molar concentration of antagonist which reduces the maximum to one half. This was intended as an experimental measure for use with all antagonists which reduce the maximum, independently of their mode of action. Only in a special case has pA_h theoretical significance, namely when the antagonism is noncompetitive and the response directly proportional to the fraction of receptors activated. In that case

$$pA_h=\log K'_2$$

In the case of a noncompetitive antagonism with a non-linear relation between activated receptors and response, the pA_2-pA_h difference varies according to the fraction of receptors activated. The relation of pA_2-pA_h and y for this case is shown in Fig. 3 (curve **b**).

Ariens and van Rossum (1957) have independently introduced a different nomenclature in which the logarithm of the affinity constant of a noncompetitive antagonist is designated as pD'_2. The two nomenclatures need not conflict if pD'_2 is used to denote an affinity constant and pA_h to denote an empirical measurement.

RESULTS

Antagonism of Pyridylethylamine and Antihistamines

β-2-Pyridylethylamine produces a variety of histamine-like effects (Walter, Hunt, and Fosbinder, 1941); it contracts the guinea-pig

TABLE I

THE pA_2 VALUES OF MEPYRAMINE AND DIPHENHYDRAMINE WITH HISTAMINE AND PYRIDYLETHYLAMINE USING THE GUINEA-PIG ILEUM

14 min. contact.

	Mepyramine	Diphenhydramine
Histamine	9·3, 9·2 } 9·3	8·3, 8·1, 7·9, 7·8, 7·6 } 7·9
Pyridylethylamine ..	9·4, 9·2, 9·2, 9·1, 9·2 } 9·2	8·2, 8·0, 7·9 } 8 0

TABLE II

ACTIVITY RATIO OF HISTAMINE AND PYRIDYLETHYLAMINE AS MEASURED IN 2 + 2 ASSAYS BEFORE AND AFTER ADDITION OF DIPHENHYDRAMINE USING THE GUINEA-PIG ILEUM

No. of Expt.	Activity Ratio Before Diphenhydramine	Activity Ratio in Presence of Diphenhydramine ($1:300\times10^4$)
1	48	57
2	39	43
3	28	31
4	46	46
5	40	41
6	65	65

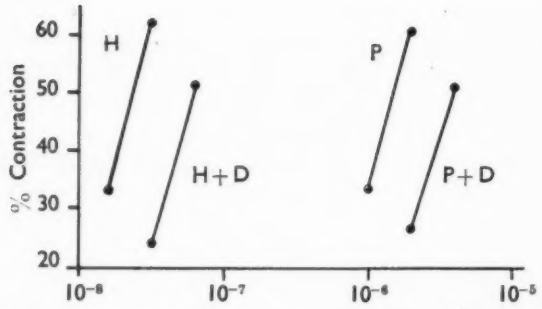

FIG. 4.—Guinea-pig ileum. Effects of histamine (H) and pyridylethylamine (P) in absence and presence of diphenhydramine (D) $1:300\times10^{6}$. Mean responses of six assays. Abscissa: concentration of agonist.

ileum, but is about 30 times less active in this respect than histamine. The object of the following experiments was to find out whether pyridylethylamine and histamine produce similar pA_x values with antihistamines.

The pA_2 of two different antihistamine drugs was measured on the guinea-pig ileum using both agonists as stimulants. Table I shows that the antihistamine drugs gave closely similar pA_2 values with the two agonists. In another set of experiments, an indirect method of comparing pA_x values was employed, in which the activity ratios of the two agonists were determined before and after administration of diphenhydramine. A typical experiment consisted of a 2+2 assay to determine the activity-ratio of histamine and pyridylethylamine, followed by a second assay with double doses of agonists in the presence of $1:300\times10^{6}$ diphenhydramine. In six such assays, no significant change in the activity ratio occurred after diphenhydramine (Table II). Fig. 4 represents the average responses in this series in terms of the maximal response. It is seen that the regression lines of the two agonists are equally displaced by the antagonist, confirming that histamine and pyridylethylamine give the same pA_x values with diphenhydramine.

Measurements of pA_x *on Perfused Lung, Isolated Tracheal Chain, and Ileum*

A set of measurements of pA_2 and pA_{10} on the air perfused lung is shown in Fig. 5. Fig. 5*a* shows the effects of 2.5 μg. histamine and Figs. 5*b-c* the effects of 5 and 25 μg. of histamine administered after various concentrations of pethidine had been added to the stock solutions. Each dose of histamine was repeated three times, and the mean area under the second and third response curve used for the calculation of pA_2 and pA_{10} values. The results obtained were: $pA_2=6.09$ and $pA_{10}=4.88$.

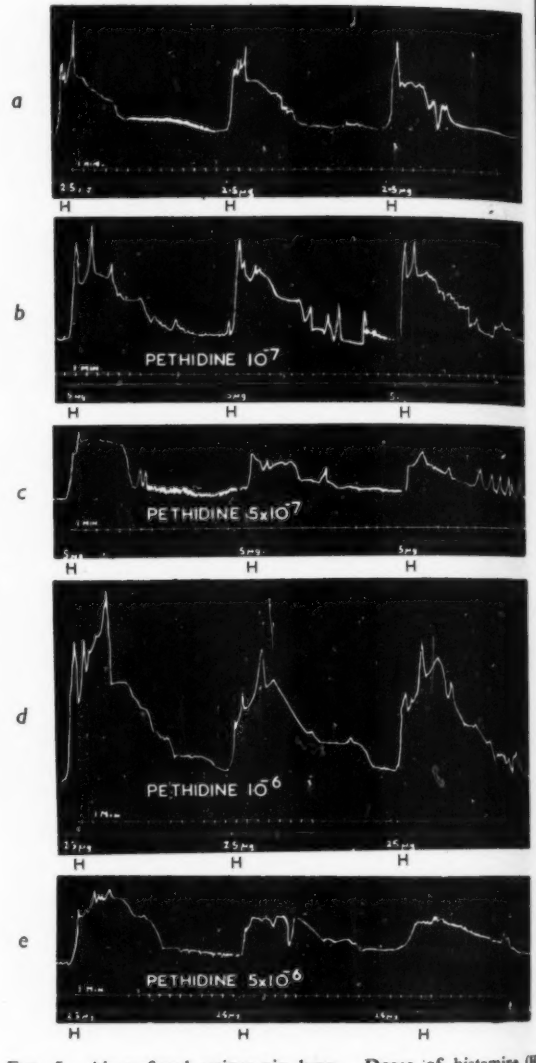

FIG. 5.—Air-perfused guinea-pig lung. Doses of histamine (H) injected into the perfusion fluid in absence and presence of pethidine hydrochloride (P). (*a*) 2·5 μg. H; (*b*) 5 μg. H with 10^{-7} P; (*c*) 5 μg. H with 5×10^{-7} P; (*d*) 25 μg. H with 10^{-6} P; (*e*) 25 μg. H with 5×10^{-6} P.

A series of similar experiments with mepyramine, diphenhydramine, pethidine, and atropine as histamine antagonists and atropine as acetylcholine antagonist are summarized in Table III, which also includes the results of earlier experiments on the guinea-pig ileum. It is seen that in spite of the difference in preparation and experimental procedure the two sets of experiments gave on the whole similar results; the differences were not statistically significant by the t-test.

TABLE III

COMPARISON OF pA_x VALUES OBTAINED ON THE GUINEA-PIG ILEUM AND THE AIR-PERFUSED LUNG

The total number of individual determinations is given in parentheses with the standard deviation. The results on the ileum are from Schild (1947a).

Active Drug	Antagonist	pA_2		pA_{10}	
		Ileum 14 min.	Air-perfused Lung 30 min.	Ileum 14 min.	Air-perfused Lung 30 min.
Histamine	Mepyramine ..	9·46 (5, 0·22)	9·37 (3, 0·16)	8·36 (1)	8·38 (3, 0·28)
	Diphenhydramine	8·02 (9, 0·28)	7·80 (4, 0·22)	7·02 (4, 0·31)	6·92 (3, 0·74)
	Pethidine	6·13 (5, 0·46)	6·15 (2, 0·08)	5·00 (5, 0·21)	5·06 (4, 0·23)
	Atropine	5·64 (3, 0·18)	5·91 (4, 0·61)	4·60 (3, 0·24)	5·03 (3, 0·68)
Acetylcholine	,, ...	8·61 (11, 0·15)	8·76 (2, 0·63)	8·05 (3, 0·13)	7·63 (6, 0·64)

It was of interest to compare the effective concentrations of both agonist and antagonist in two tissues. Since in the perfused lung preparation the agonist was injected and its final concentration was undefined, we used the isolated guinea-pig tracheal chain (Castillo and de Beer, 1947) and ileum for this comparison. Table IV shows the effective doses of histamine, expressed in terms of log concentration giving a 50% response, and the pA_2 values of diphenhydramine and mepyramine in the two preparations. It is seen that whilst the pA_2 values are similar the effective concentrations of histamine in the two tissues vary. It follows that the drug ratios (agonist/antagonist, Gaddum *et al.*, 1955) in the two tissues are also different.

TABLE IV

EFFECTIVE CONCENTRATIONS OF HISTAMINE AND ANTAGONISTS IN TWO PREPARATIONS

The number of experiments is given in brackets.

	$-\log ED50$ Histamine	pA_2	
		Diphenhydramine	Mepyramine
Ileum	7·7 (11)	7·9 (5)	9·3 (2)
Tracheal chain ..	6·1 (4)	7·8 (2)	9·1 (2)

Antagonism Acetylcholine-atropine

Previous estimates of $pA_2 - pA_{10}$ for acetylcholine-atropine gave lower estimates than the theoretical value of 0.95 to be expected for a simple competitive antagonism at equilibrium. We have reinvestigated this antagonism employing a wider range of concentrations of atropine and as far as possible under equilibrium conditions.

In assessing the significance of the low $pA_2 - pA_{10}$ values in earlier work (Schild, 1947a: 0.56; Marshall, 1955b: 0.73) two possible sources of error have to be taken into account. (1) Failure to attain equilibrium. This applies particularly to the measurements of Schild (1947a) which were taken after a fixed period of 14 min. contact with antagonist. (2) Paradoxical potentiating effects. Very low concentrations of atropine sometimes produce a potentiation instead of antagonism and this would interfere with pA_2 measurements.

Fig. 6 illustrates an experiment in which a wide range of concentrations of atropine was employed. In this instance hexamethonium (10^{-6}) was added to the bath fluid in order to prevent stimulation of the ganglia of Auerbach's plexus by high concentrations of acetylcholine. Before adding atropine the preparation was stabilized by administering about 100 doses of acetylcholine. Only the middle range of the response curve was utilized and each point in Fig. 6 represents the mean of several responses. The log dose/effect curves are parallel, though steeper than would be expected from simple proportionality between receptors and response. When plotted by using equation (1), the results can be fitted by a straight line with slope $-n = 1.04$ (Fig. 7).

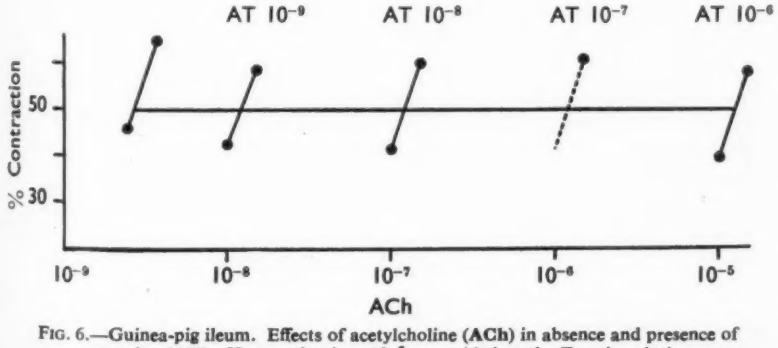

FIG. 6.—Guinea-pig ileum. Effects of acetylcholine (ACh) in absence and presence of atropine (AT). Hexamethonium 10^{-6} was added to the Tyrode solution.

In a series of experiments with two or more doses of atropine, the following simplified procedure was adopted: a stable submaximal response was first produced with acetylcholine alone and reproduced afterwards with a larger dose of acetylcholine in the presence

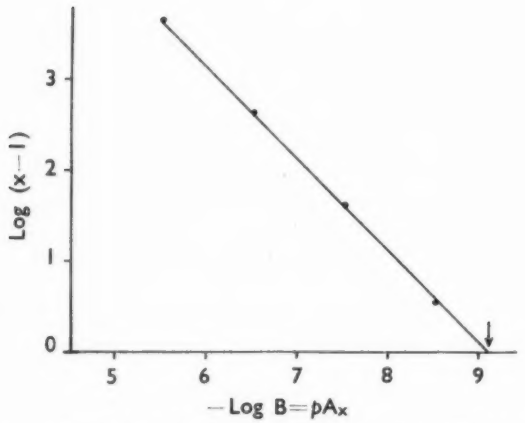

FIG. 7.—Results from Fig. 6 plotted by using equation (1) developed in the text $-n=1{\cdot}04$. The arrow indicates the pA_2 value.

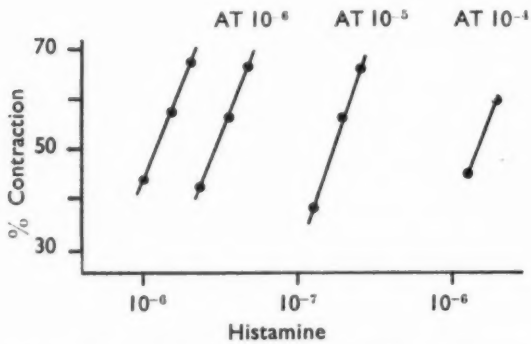

FIG. 8.—Guinea-pig ileum. Effects of histamine in absence and presence of atropine (AT).

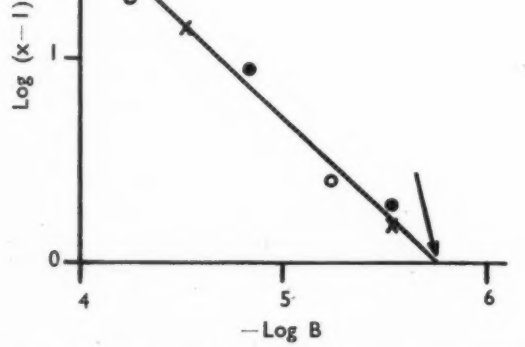

FIG. 9.—Plot of equation (1) for three separate experiments on guinea-pig ileum, with histamine as agonist and atropine as antagonist. The arrow indicates the pA_2 value.

of atropine. For each response in the presence of atropine a value of K_2 for $n=1$ was calculated, and this is shown in Table V. Omitting one discrepant result (Expt. No. 8), Table V shows no significant change in the value of log K_2 within each experiment ($P>0.7$). It can be concluded that K_2 is a constant and that the results are compatible with the hypothesis of a simple competitive antagonism with $n=1$, in which case log $K_2=pA_2$. The calculated average equilibrium value of pA_2 is 9.0, which is somewhat higher than the earlier value of 8.61 obtained by the standard method after 14 min. contact with atropine.

Antagonism Histamine-atropine

Atropine is a weak histamine antagonist, being about 1,000 times less active against histamine than against acetylcholine (Schild, 1947a). Nevertheless the antagonism between histamine and atropine is probably not unspecific but competitive.

The log dose/effect curves of histamine with atropine are parallel over a considerable range (Fig. 8), though very high concentrations of atropine (10^{-4}) tend to flatten the slope. Fig. 9 shows a plot of equation (1) which embodies the results of three separate experiments. The experimental points are fitted by a straight line with a slope ($-n$) of 0.92. The graphical pA_2 value is 5.76 as against 5.64 in earlier work.

TABLE V

VALUES OF LOG $\frac{X-1}{B}$ = LOG K_2 FOR ACETYLCHOLINE-ATROPINE ON GUINEA-PIG ILEUM

In Expt. Nos. 1 and 2 hexamethonium 10^{-6} was added to the Tyrode solution.

No. of Expt.	−Log Conc. Atropine Sulphate	Log K_2	
1	9	9·06	
	8	9·16	
	7	9·15	
	6	9·17	9·14
2	9	9·07	
	6	9·06	9·07
3	9	9·0	
	8	8·87	
	7	8·9	8·92
4	9·1	8·81	
	8·1	8·88	8·85
5	9·1	9·1	
	8·1	9·04	9·07
6	9·3	8·83	
	8·3	8·74	8·79
7	9·5	9·03	
	8·53	9·01	9·02
8	10·0	9·53	
	8·62	9·1	9·32
9	9·28	8·81	
	8·42	8·9	8·86
			9·0

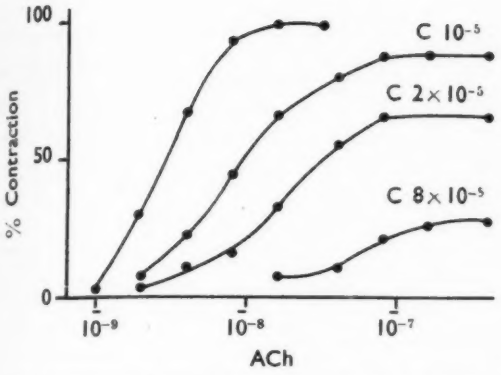

FIG. 10.—Guinea-pig ileum. Dose/response curves of acetylcholine (ACh) with cinchonidine (C). The left-hand curve was in the absence of cinchonidine.

Atropine thus behaves as an imperfect competitive antagonist of histamine over a certain range presumably by virtue of a weak affinity for histamine receptors.

Antagonism Acetylcholine-cinchonidine

Fig. 10 shows log dose/effect curves of acetylcholine with cinchonidine obtained on the guinea-pig ileum. The slope of the curves becomes progressively flatter and their maxima decline; in the end all doses of acetylcholine become ineffective. Usually the flattening of the slope precedes the diminution of the maximum. Very high concentrations of cinchonidine also alter the character of the response to a brief contraction followed by relaxation. Similar curves can also be obtained with histamine as agonist.

Sometimes a parallel shift of the log dose/effect curves precedes the flattening of the slope, but we found it difficult to decide whether this was a true effect or an artifact due to persistent depression from an earlier large dose. In order to avoid this kind of artifact the measurements were generally so conducted that maximal effects were elicited only at the beginning; the preparation was then allowed to recover and the subsequent responses elicited in the middle range. Under these conditions cinchonidine regularly produced a flattening of the slope as shown in Fig. 11.

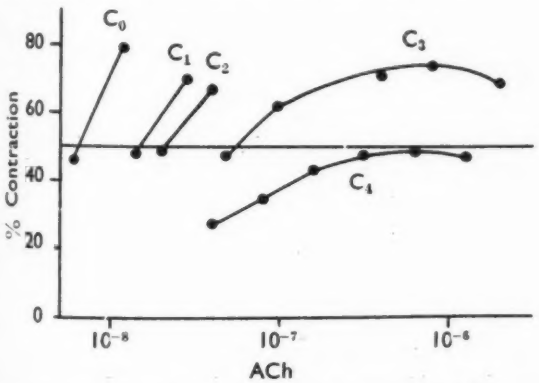

FIG. 11.—Guinea-pig ileum. Short dose/response curves of acetylcholine (ACh) with cinchonidine (C): C_0=no cinchonidine, $C_1=5\times10^{-8}$, $C_2=10^{-5}$, $C_3=2\times10^{-5}$, $C_4=4\times10^{-5}$.

The pA_2 was measured at the 50% response level from curves such as those shown in Fig. 11. The final value was obtained by interpolation or close extrapolation. The pA_h was obtained by interpolation or close extrapolation from two curves whose maxima were reduced to near 50%. To identify the maximum, doses of acetylcholine were successively doubled and the maximum was considered to have been reached when a doubling of the dose produced either no increase or a decrease in response. Values of K'_2 were calculated from equation (2) for each set of two consecutive responses in the presence of cinchonidine.

The results of these measurements are summarized in Table VI. It is seen that the pA_h values are more consistent than the values of log K'_2 which vary within and between experiments. The average pA_2–pA_h difference is 0.9, which in a noncompetitive antagonism corresponds to 14% receptor activation for a 50% response (Fig. 3**b**). However, in view of the atypical responses obtained in the presence of cinchonidine and the variability of K'_2, it is doubtful that a true noncompetitive antagonism can be assumed, and it would seem more justified to regard pA_h in this instance simply as an

TABLE VI

ACETYLCHOLINE-CINCHONIDINE. GUINEA-PIG ILEUM

No. of Expt.	Log K'_2		pA_2	pA_h	pA_2–pA_h
1	4·6				
	4·9				
	4·2	4·6	4·7	3·8	0·9
2	5·5				
	4·8	5·2	4·9	4·0	0·9
3	5·0				
	5·1				
	5·1	5·1	5·1	3·9	1·1
4	4·8				
	5·0				
	4·9	4·9	5·0	4·0	1·0
5	3·9				
	4·4	4·2	4·4	4·0	0·4
6	5·1				
	4·7				
	4·7	4·8	5·0	4·0	1·0
7	4·9				
	4·8				
	4·8	4·9	4·9	3·9	1·0
		4·8	4·9	4·0	0·9

experimental measurement. The pA_h can then be regarded as a measure of the antagonistic activity of cinchonidine and the pA_2-pA_h as a measure of the main effective range of the antagonist.

Discussion

The various quantitative applications of pA_x described in this paper are all based on the hypothesis which Gaddum (1937) formulated mathematically, that agonists and antagonists compete for receptors according to the mass law. This hypothesis, although no doubt oversimplified, accounts surprisingly well for a variety of experimental findings.

Use of pA_x for the Classification of Drugs

Agonists which act on the same receptors can theoretically be expected to produce the same pA_x with competitive antagonists. This is a consequence of the mass law and applies whatever the affinity of the agonist or its intrinsic activity (Ariens, 1954) or efficacy (Stephenson, 1956). If it could be verified experimentally that certain groups of agonists produce the same pA_x (or dose ratio) with antagonists, the receptor theory would be strengthened and a precise method provided of classifying agonists according to the receptors on which they act (Schild, 1947b). By this criterion histamine and pyridylethylamine would be acting on the same receptors but not histamine and acetylcholine, because although they are both antagonized by atropine their pA_x values are different.

Very few quantitative investigations of this kind are available and it is not known how far the principle of classifying agonists by their pA_x values can be extended ; it tends to break down, however, when applied to agonists with an indirect action. Thus atropine gives similar (though not identical) pA_x values with acetylcholine and tetramethylammonium (Clark and Raventos, 1937), but it need not follow that the two agonists act on the same primary receptors. The similarity of their pA_x values may be due to tetramethylammonium causing a release of acetylcholine which is then antagonized by atropine.

Use of pA_x for the Comparison of Receptors

Table VII gives a summary of pA_x values of atropine and antihistamines in different preparations. The values in different preparations are on the whole remarkably similar. Thus in the case of atropine similar pA_x values are found in such varied preparations as frog heart, chick amnion, and mammalian intestine, the one exception being the frog rectus which presumably has different, nicotinic receptors. The finding that different tissues have receptors with similar affinities for antagonists is interesting since it gives support to the notion that receptors are definite chemical entities.

TABLE VII

pA_x VALUES IN DIFFERENT PREPARATIONS

(1) Clark and Raventos (1937). (2) De Jalon (after Reuse, 1948). (3) Evans and Schild (unpublished observations). (4) Hawkins and Schild (1951). (5) Marshall (1955a). (6) Marshall (1955b). (7) Reuse (1948). (8) Schild (1947a). (9) Present work. (10) Wilbrandt (1950).

Preparation	Atropine-acetylcholine		Mepyramine-histamine	Diphenhydramine-histamine
	pA_2	pA_{10}	pA_2	pA_2
Guinea-pig ileum ..	8·8 { 8·6 (8), 9·0 (9), 8·8 (6)	8·1 { 8·1 (8), 8·1 (6)	9·3 { 9·5 (8), 9·3 (7), 9·3 (2), 9·4 (5), 9·3 (9)	8·0 { 8·0 (8), 8·2 (2), 7·8 (10), 8·1 (5)
Rat intestine ..		8·1 (1)		
Guinea-pig trachea ..			9·1 (9)	7·8 (9)
Guinea-pig lung (perfused) ..	8·8 (9)	7·6 (9)	9·4 (9)	7·8 (9)
Human bronchi			9·3 (4)	
Chick amnion	8·8 (3)			
Frog auricle ..		8·3 (1)		
,, rectus ..		4·2 (1)		

Criticism of Theory of Competitive Antagonism

Although atropine seems to behave as a competitive antagonist of acetylcholine over a wide range of concentrations, there is an old objection against the view that this is a competitive antagonism which was first raised by Clark (1926). It arises from the apparent slow dissociation of atropine from its receptors which makes it difficult to understand that acetylcholine can produce immediate large effects in fully atropinized preparations. One way of formulating this objection is as follows: the very notion of competitive antagonism implies an equilibrium, therefore if acetylcholine cannot get immediately at the receptors its observed effects do not represent an equilibrium and cannot be made the basis of a test for competitive antagonism. Several ways of resolving this difficulty can be proposed.

(a) The antagonism is noncompetitive, and the apparent agreement with competitive antagonism arises from the fact that only few receptors are occupied by the agonist, in which case competitive

and noncompetitive antagonists become indistinguishable. It is possible to calculate on this assumption the maximum fraction of receptors that could be occupied by acetylcholine for a given experimental result. This fraction turns out to be exceedingly small. For instance, in order to account in terms of noncompetitive antagonism for the results of experiments with a thousandfold range of atropine (Table V), only about 1/10,000th of receptors could have been occupied by acetylcholine.

(b) The antagonism is competitive in the sense that acetylcholine and atropine act on the same site, but owing to the slow rate of dissociation of atropine from its receptors only the initially unoccupied receptors are immediately available to acetylcholine. Acetylcholine nevertheless can produce effects by combining with a very few "spare" receptors. This is the hypothesis of Stephenson (1956) which has an interesting consequence pointed out by Furchgott (1955). The antagonism would then be practically noncompetitive as regards immediate effects of acetylcholine in an atropinized preparation, since the receptors occupied by atropine would be as unavailable as if blocked by a noncompetitive antagonist. In the limiting case of a comparatively very slow rate of association and dissociation of atropine, the same mathematical consequences would follow as in noncompetitive antagonism and the fraction of receptors occupied by acetylcholine would be as small, or nearly as small, as in (a) above.

(c) The antagonism is competitive and equilibria at the receptor level are rapidly established, but atropine lingers on in a hypothetical "biophase" (Ferguson, 1939; Fastier and Reid, 1948; Furchgott, 1955) which determines the rate of onset and disappearance of its action. The biophase presumably represents the immediate environment of the receptors and not the intracellular spaces, since del Castillo and Katz (1957) have shown that the effects of curare are delayed even when it is applied directly to the motor end plate by a micropipette.

(d) Acetylcholine actually displaces atropine from receptors. This hypothesis has often been advocated, although it is incompatible with the mass law. Clark (1926) tested it experimentally by trying whether a large dose of acetylcholine would speed up the removal of atropine, but obtained no such effect.

In summary, if the antagonism is to be competitive it is necessary to postulate either a diffusion barrier for atropine as suggested by Furchgott (1955) or a very small fraction of receptors occupied by acetylcholine as suggested by Stephenson (1956) or some combination of the two effects. The issue thus hinges largely on the fraction of receptors occupied by acetylcholine.

Noncompetitive Antagonism

The fraction of receptors occupied by acetylcholine could in theory be determined with the aid of a noncompetitive antagonist. Unfortunately a reversible noncompetitive antagonist which obeys the mass law quantitatively does not seem to have been found so far and the following considerations suggest that such an antagonist may be difficult to find.

The antagonist could act in two ways: (1) By reversible inactivation of one kind of receptor; this would constitute a specific noncompetitive antagonism. But experience so far has shown that antagonists which inactivate receptors produce irreversible effects; they behave as "non-equilibrium" antagonists (Nickerson, 1957). (2) By reversible inactivation of a later reaction stage; this would constitute an unspecific noncompetitive antagonism. The effects of unspecific antagonists may, however, be too complicated to be formulated in terms of the mass law. Shanes (1958) has suggested that these antagonists may penetrate the cell membrane and interfere with ionic fluxes during excitation. They would then produce their effects by increasing the spreading tendency of the cell membrane and narrowing the pores through which ions pass. A similar mode of action was suggested by Skou (1954) for local anaesthetics.

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SOME PHARMACOLOGICAL PROPERTIES OF THE α-TOXIN OF *STAPHYLOCOCCUS PYOGENES*

BY

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The α-toxin of *Staphylococcus pyogenes* produced a slowly developing contracture of isolated preparations of rabbit jejunum and of guinea-pig ileum which persisted after thorough washing and left the gut unresponsive to further doses of α-toxin or of acetylcholine. After incubation with antitoxin, the α-toxin no longer produced a contracture. Antitoxin only prevented the α-toxin response if added to the bath fluid before but not after the α-toxin. Certain drugs reduced the α-toxin contracture when added to the bath fluid before or after the α-toxin, but the contracture reappeared on washing. Papaverine abolished the contracture and pethidine was only slightly less active. Mepyramine, amyl nitrite, caffeine, aminophylline, adrenaline and ephedrine partly reduced the contracture. Hexamethonium, cocaine, tubocurarine and gallamine had no effect. The effect of atropine was only small. The gut-stimulant activity/haemolytic unit of two α-toxin samples differed greatly; this difference did not appear to be due to activity of impurities. The implications of these observations are discussed.

Certain strains of *Staphylococcus pyogenes*, including those causing food-poisoning outbreaks in man, produce toxins which stimulate gut *in vitro*. Feldberg and Kellaway (1938) first described the action of staphylococcal toxin on isolated guinea-pig ileum, and Richmond, Reed, Shaughnessy, and Michael (1942) described its action on rabbit intestine. Anderson, James, and Marks (1954) concluded that the response of isolated rabbit jejunum to extracts of *S. pyogenes* cultures was determined mainly by their α-toxin content, that is the α-haemolytic fraction.

The present work was mainly undertaken to study further the responses of isolated segments of rabbit jejunum and guinea-pig ileum to the α-toxin of *S. pyogenes*, and to examine how far pharmacological agents could modify these responses.

METHOD

Segments of rabbit jejunum were suspended in Ringer-Locke solution at 37° in a 30 ml. bath and bubbled with pure oxygen. Segments of guinea-pig mid-ileum were usually suspended in Tyrode solution at 30° in a 30 ml. bath and bubbled with air. Contractions were recorded on smoked paper with frontal writing levers giving a 5-fold magnification.

The irreversible nature of the contracture in response to the α-toxin precluded repetitive testing on the same preparation. Therefore, the following methods were used.

Method 1.—Two adjacent lengths of gut were set up in separate baths. Their responses to a reference dose of acetylcholine were recorded. To one bath the α-toxin was added and the height of the contracture was compared with the response to the dose of acetylcholine. 2 min. before adding the same dose of α-toxin to the other bath, the antagonist was administered. The height of the contracture produced by the α-toxin was compared with that following the dose of acetylcholine.

Method 2.—A single preparation was used and, in order to determine the antagonistic effect of drugs on the α-toxin contracture, the antagonists were added to the bath 2 min. before the α-toxin. The ensuing contracture was then recorded in the presence of the antagonist and later after washing the preparation. If the antagonist abolished the contracture, the preparation was washed to see if the contracture developed subsequently.

Method 3.—A single preparation was used, but a contracture was first elicited with the α-toxin and the antagonist was added later.

Materials.—Three batches of α-toxin (K 9634, K 160, and K 782) were obtained from the Wellcome Research Laboratories. They were filtrates from staphylococcal cultures grown on a sloppy nutrient agar medium, clarified by centrifugation. The toxin was precipitated by $(NH_4)_2SO_4$, the precipitate redissolved in M/15-borate buffer and freeze-dried. In this manner the agar and low molecular-weight constituents of the culture fluid were removed, but the

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material of high molecular weight remained. The product was dissolved in distilled water immediately before adding to the preparations and had a haemolytic potency of about 3.5 haemolytic units/mg. A haemolytic unit (H.U.) is the smallest amount of α-toxin producing complete haemolysis of 1 ml. of a 1% suspension of fresh washed rabbit erythrocytes in normal saline. Unless otherwise stated, the Wellcome α-toxin was employed.

In a few experiments a purified α-toxin, kindly given us by Dr. L. Butler, was used. This was an $(NH_4)_2SO_4$ precipitate with a haemolytic potency of 75 H.U./mg. of dried powder.

A sample of uninoculated culture medium similar to that used in the preparation of the Wellcome α-toxin samples was used as a control. This medium was treated in the same way as the inoculated medium, with the exception of freeze-drying. The amount of culture medium residue present in 1 ml. of this sample was approximately equivalent to that in 10 H.U. of the Wellcome α-toxin samples.

A staphylococcal antitoxin was used which was a peptic-refined horse serum containing 1,250 units of antitoxin/ml. (Wellcome Research Laboratories, Serial No. RA 318A).

Acetylcholine chloride, histamine acid phosphate, cocaine hydrochloride, amyl nitrite, nicotine hydrogen chloride (Savory and Moore), hexamethonium tartrate, gallamine triethiodide, mepyramine maleate (May and Baker), adrenaline acid tartrate, tubocurarine chloride (Burroughs Wellcome), caffeine sodium salicylate (British Drug Houses), pethidine hydrochloride (Roche), atropine sulphate, ephedrine sulphate, aminophylline, and papaverine hydrochloride were used, and doses are expressed as concentrations of salt added to the bath.

RESULTS

Doses of between 0.03 and 0.3 H.U. of α-toxin/ml. produced a slowly-developing contracture in both rabbit jejunum and guinea-pig ileum (Figs. 1, 2*b*, and 7*b*). In the rabbit jejunum at 37°, there was a delay varying from a few seconds to 2 min. between the addition of α-toxin and the onset of contracture, and a further 1 to 2 min. elapsed before the contracture reached maximum. In the corresponding experiments on the guinea-pig ileum suspended at 37°, the latency was 1 to 2 min., and the maximum of the contracture was reached 2 to 5 min. later. When the guinea-pig ileum was suspended at 30°, the latency was 4 to 5 min., and full development was reached after another 5 to 15 min. Thus the guinea-pig ileum appeared more sluggish in responding to the α-toxin than did the rabbit jejunum.

The spontaneous rhythmic contractions recorded from the rabbit jejunum initially increased on adding the α-toxin, but decreased as the general tone of the gut rose. In the guinea-

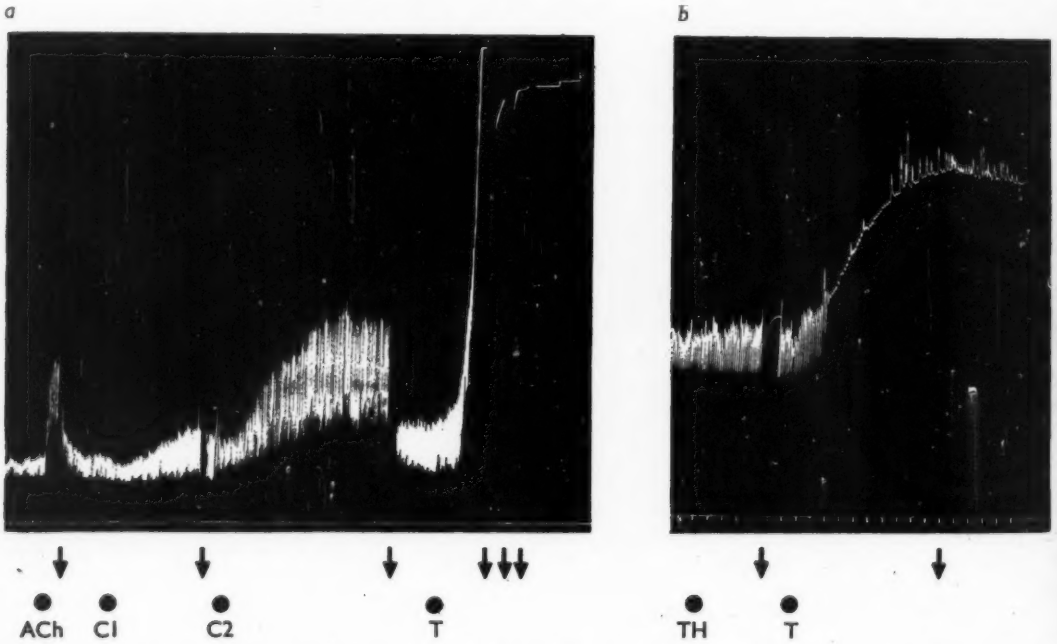

FIG. 1.—Two isolated rabbit jejunum preparations (*a* and *b*) in Ringer-Locke solution. (*a*) Effects of uninoculated culture medium, the amounts being those contained in 10 (at C1) and 20 (at C2) H.U. of α-toxin, and of 10 H.U. of α-toxin (at T). ACh, 0.1 μg. of acetylcholine. (*b*) Effect of 5 H.U. of α-toxin before (at T) and after heating to 80° for 45 min. (at TH). Arrows indicate washing. Time, 30 sec. Bath volume, 30 ml.

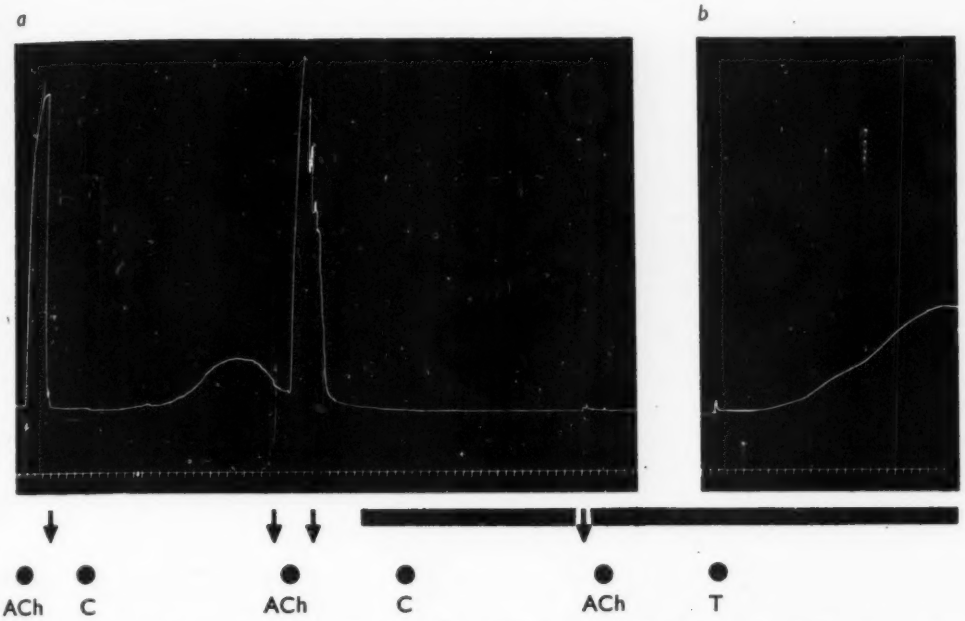

FIG. 2.—Isolated guinea-pig ileum preparation in Tyrode solution. At **C**, uninoculated culture medium, the amount being that contained in 20 H.U. of α-toxin; at **T**, 20 H.U. of α-toxin; at **ACh**, 0.1 μg. of acetylcholine. The horizontal line indicates the presence of 10 μg. of atropine. There was an interval of 10 min. between (*a*) and (*b*). Arrows indicate washing. Time, 30 sec. Bath volume, 30 ml.

pig ileum, spontaneous contractions were absent or small.

After washing, the α-toxin contracture persisted and was frequently increased in both species; occasionally it was reduced but never abolished. When α-toxin or acetylcholine were then added to the bath, there was usually no response. A small response to acetylcholine was sometimes observed particularly in preparations in which washing had produced partial relaxation (Fig. 3). Even in these preparations, the acetylcholine sensitivity, when tested at about 10 min. intervals, decreased progressively.

When α-toxin was heated to 80° for 45 min. or to 100° for 10 min., it no longer caused contracture of the rabbit or guinea-pig intestine. Fig. 1*b* illustrates the ineffectiveness of 5 H.U. of heated α-toxin (at **TH**).

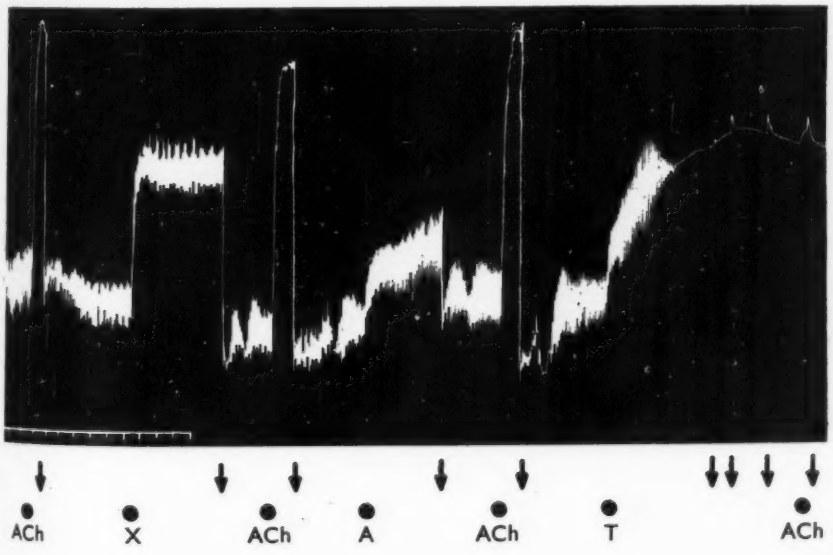

FIG. 3.—Isolated rabbit jejunum preparation in Ringer-Locke solution. At **ACh**, 1 μg. of acetylcholine; at **X**, a mixture of 25 units of antitoxin and 10 H.U. of α-toxin; at **A**, 25 units of antitoxin; at **T**, 10 H.U. of α-toxin. The arrows indicate washing. Time, 1 min. Bath volume, 30 ml.

The action of α-toxin on the intestine may be an indirect effect involving the release of a muscle-stimulating substance (Feldberg and Kellaway, 1938). However, it was not possible to obtain evidence for such a release. For instance, in several experiments, 20 H.U. of α-toxin in 1 ml. of Tyrode solution were placed inside the closed lumen of segments of guinea-pig ileum which were then immersed for 1 hr. in tubes containing 5 ml. glucose-free Tyrode solution at 37°. Control loops containing 1 ml. Tyrode solution alone were set up under identical conditions. The segments were then removed and the fluid was tested on fresh preparations either directly or after 24 hr. dialysis (two experiments). In no experiment was any difference found between control and test fluids. Both produced a contracture which was readily reversed by washing. The contracture was not modified by mepyramine and atropine in doses sufficient to prevent the much larger responses to 33 ng./ml. of histamine and to 33 ng./ml. of acetylcholine. Thus, although dialysable, the substance or substances released in the test or control experiments did not appear to be histamine or acetylcholine and their presence in the fluid was independent of the α-toxin.

Effect of Spasmolytic Agents

Rabbit Jejunum.—Pethidine and papaverine in concentrations of 33 μg./ml. rendered the rabbit intestine insensitive or nearly insensitive to the action of 4 H.U. of α-toxin as long as the spasmolytics were kept in the bath fluid. This is shown for pethidine in the experiment illustrated in Fig. 4 and for papaverine in the experiment in Fig. 5. When the bath fluid was then replaced with fresh solution, the α-toxin contracture

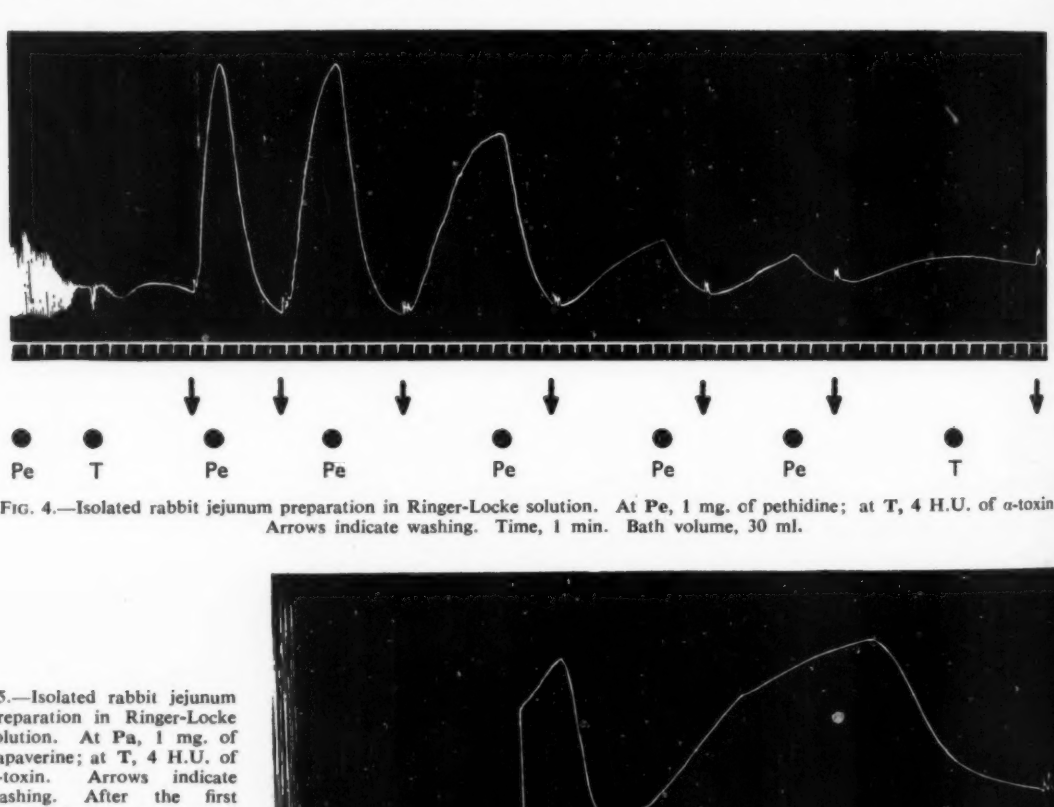

FIG. 4.—Isolated rabbit jejunum preparation in Ringer-Locke solution. At **Pe**, 1 mg. of pethidine; at **T**, 4 H.U. of α-toxin. Arrows indicate washing. Time, 1 min. Bath volume, 30 ml.

FIG. 5.—Isolated rabbit jejunum preparation in Ringer-Locke solution. At **Pa**, 1 mg. of papaverine; at **T**, 4 H.U. of α-toxin. Arrows indicate washing. After the first washing the drum was stopped for 3 min. Time, 30 sec. Bath volume, 30 ml.

developed but could again be abolished by the renewed addition of the spasmolytic. When this procedure was repeated several times, the contracture on replacing the bath fluid with fresh solution became progressively smaller. The preparation was then insensitive to α-toxin as well as to acetylcholine.

Adrenaline, ephedrine, amyl nitrite, mepyramine, caffeine and aminophylline reduced but rarely abolished the α-toxin spasm when added to the bath fluid in concentrations of 33 μg./ml. The effect lasted only as long as the spasmolytics were kept in the bath, and with adrenaline the effect even disappeared if the adrenaline was kept in the bath fluid and the preparation contracted after 2 to 4 min. Ephedrine produced relaxation of the α-toxin spasm in concentrations 5 to 10 times as large as adrenaline, and the relaxation was maintained as long as the ephedrine was kept in the bath fluid.

Tubocurarine, gallamine, and hexamethonium had no effect on the α-toxin contracture, nor had atropine except in one experiment in which 33 ng./ml. caused a transient relaxation of the α-toxin spasm. When the contracture reappeared the addition of 33 ng./ml. of atropine did not cause a renewed relaxation.

A comparison of the spasmolytic potency of the various drugs on the α-toxin contracture in rabbit intestine is shown in Table I, which gives the approximate % reduction of the α-toxin spasm. Each value is the mean of three to five experiments.

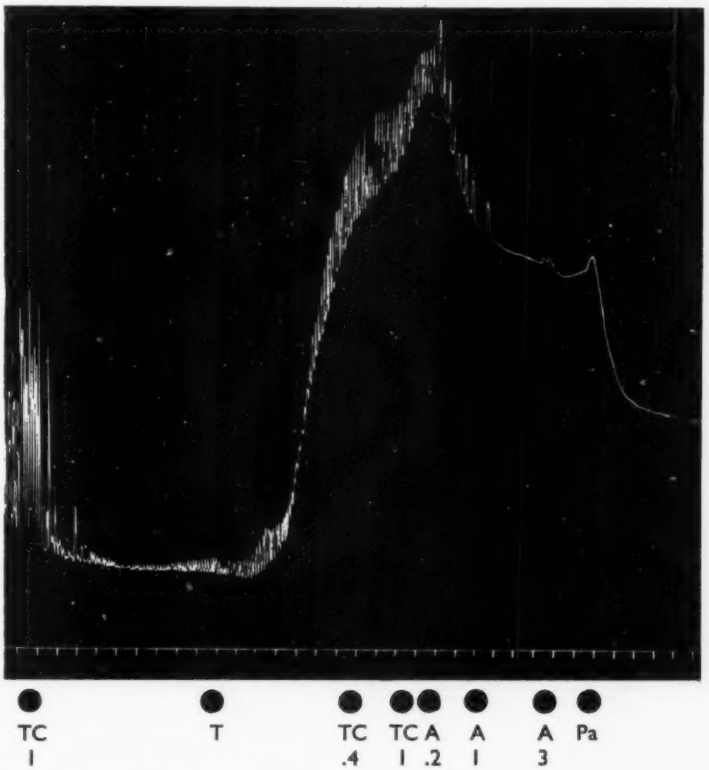

FIG. 6.—Isolated rabbit jejunum preparation in Ringer-Locke solution. At **TC1** and **TC.4**, 10 and 4 mg. respectively of tubocurarine; at **T**, 4 H.U. of α-toxin; **A.2**, **A1** and, **A3**, 0.2, 1, and 3 mg. respectively of adrenaline; at **Pa**, 2 mg. of papaverine. Time, 30 sec. Bath volume, 30 ml.

TABLE I

THE EFFECT OF SPASMOLYTICS ON THE CONTRACTURE OF THE ISOLATED RABBIT JEJUNUM PRODUCED BY 4 H.U. OF α-TOXIN

The numerals give the % reduction of the height of the contracture in the presence of 1 mg. of the spasmolytic in the 30-ml. bath except where otherwise indicated.

Drug	% Reduction	
	Methods 1(*) and 2	Method 3
Tubocurarine, Gallamine, Hexamethonium	0*	0
Atropine (up to 250 μg.)	0*	0
Adrenaline	40	75
Ephedrine (5 mg.)	—	35
Aminophylline	50	60
Caffeine	—	45
Amyl nitrite	80	—
Mepyramine	85	—
Pethidine	95	100
Papaverine	100	100

When the less active spasmolytics had exerted their maximum effect, the more active relaxants produced a further relaxation of the α-toxin spasm. Fig. 6 illustrates an experiment in which 0.33 mg./ml. of tubocurarine (at **TC1**) almost abolished peristalsis but did not oppose the development of a contracture in response to the subsequent addition of 4 H.U. of α-toxin (at **T**). Increases of the tubocurarine concentration, first to 0.47 and then to 0.8 mg./ml. (at **TC.4** and **TC1**), had no effect on the contracture, but the subsequent addition of adrenaline in three concentrations of 6.6, 40, and 140 μg./ml. (at **A.2, A1**, and **A3**) caused partial relaxation ; there was some further relaxation with 66 μg./ml. of papaverine (at **Pa**).

Guinea-pig Ileum.—In the presence of pethidine or papaverine in amounts of 6.6 to 8.3 μg./ml. in the bath, α-toxin produced no contracture. This is shown for papaverine in

Fig. 7*a*. As in the rabbit intestine, the antagonistic action of pethidine and of papaverine persisted only as long as these spasmolytics were in the bath fluid, and on replacing the bath fluid with fresh solution a contracture developed. When pethidine or papaverine was given at the height of an α-toxin contracture and kept in the bath, full relaxation ensued within 30 min., and the preparation remained relaxed after replacing the bath fluid with fresh solution. This is shown in Fig. 7*b*. In this condition the relaxed preparation was insensitive to α-toxin, to potassium, and to amounts of acetylcholine which would have usually produced a strong response. As is seen in Fig. 7*b*, even an amount of acetylcholine 200 times greater than that effective on the untreated preparation only caused slight stimulation.

Mepyramine and hexamethonium produced a partial relaxation of the α-toxin spasm for as long as they were kept in the bath. Adrenaline had the same effect, but its action appeared to be as transient as on the rabbit intestine.

The effect of atropine was peculiar. A concentration of 3.3 μg./ml. usually delayed the onset of the α-toxin spasm if added before the α-toxin, although the final height of the contracture did not appear to be influenced. On the other hand, when as small a quantity as 33 ng./ml. was added at the peak of an α-toxin contracture, it reduced the contracture to about half its height, but it was not possible to obtain full relaxation with 3.3 μg./ml. of atropine.

Nicotine, tubocurarine, and cocaine in concentrations up to 33 μg./ml. did not relax the α-toxin spasm in guinea-pig preparations bathed in Tyrode solution at 37°.

A comparison of the spasmolytic potency of the various drugs in relaxing the α-toxin spasm in guinea-pig intestine is given in Table II. Each value is the mean of three to five experiments.

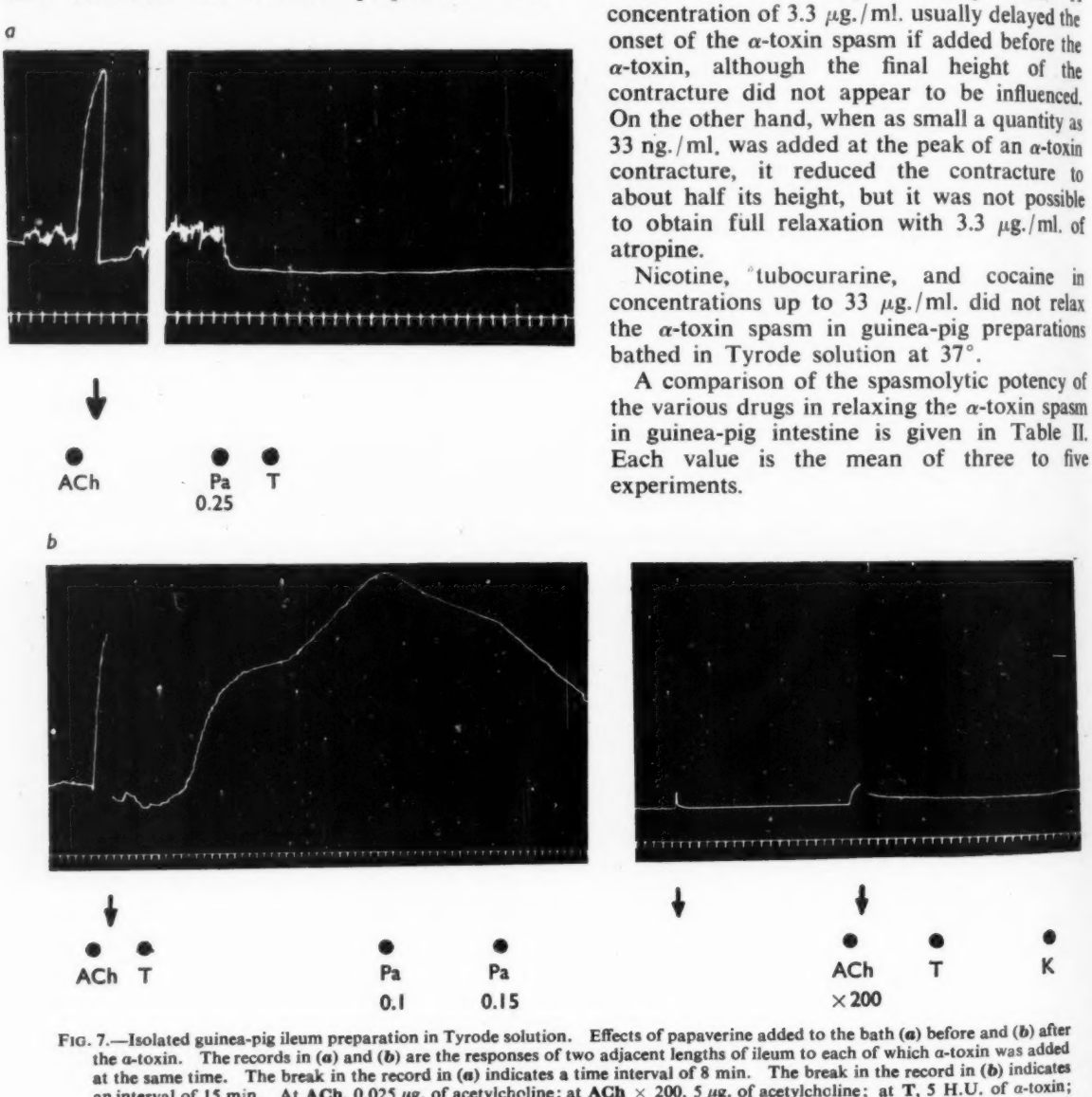

FIG. 7.—Isolated guinea-pig ileum preparation in Tyrode solution. Effects of papaverine added to the bath (*a*) before and (*b*) after the α-toxin. The records in (*a*) and (*b*) are the responses of two adjacent lengths of ileum to each of which α-toxin was added at the same time. The break in the record in (*a*) indicates a time interval of 8 min. The break in the record in (*b*) indicates an interval of 15 min. At **ACh**, 0.025 μg. of acetylcholine; at **ACh** × 200, 5 μg. of acetylcholine; at **T**, 5 H.U. of α-toxin; at **Pa** 0.1, **Pa** 0.15, and **Pa** 0.25, 0.1, 0.15, and 0.25 mg. respectively of papaverine; at **K**, 10 mg. of KCl. Arrows indicate washing. Time, 30 sec. Bath volume, 30 ml.

When the less active spasmolytics had exerted their maximum effect, the more active relaxants such as pethidine, papaverine, mepyramine, and adrenaline could produce, as in the experiments with rabbit intestine, a further relaxation of the α-toxin.

TABLE II

THE EFFECT OF SPASMOLYTICS ON THE CONTRACTURE OF THE ISOLATED GUINEA-PIG ILEUM PRODUCED BY 5 H.U. OF α-TOXIN

The numerals give the % reduction of the height of the contracture in the presence of the spasmolytic in the 30-ml. bath.

Drug	Dose (μg.)	% Reduction	
		Method 1	Method 3
Hexamethonium ..	1,000	0	20
Atropine	100	0	50
Mepyramine	200	15	50
Pethidine	200	30	80
Papaverine	100	80	90
"	250	100	100

Effect of the Antitoxin

Injection of 650 units antitoxin/kg. intraperitoneally into a guinea-pig 4 hr. prior to killing greatly reduced and delayed the contracture produced in an isolated ileal segment of that guinea-pig in response to 5 H.U. of α-toxin. Intraperitoneal injection of 300 units antitoxin/kg. produced little protection against the α-toxin spasm.

In several experiments, mixtures of α-toxin and antitoxin after incubation at 37° for 45 min. were tested on the guinea-pig ileum. Since the antitoxin alone had a strong stimulating effect of its own, it was not possible to use the heights of the contractures to measure its antagonistic effect. For instance, when the heights of the responses to antitoxin (180 units), to α-toxin (5 H.U.) and to a mixture of both were expressed as percentages of responses obtained with 0.025 μg. of acetylcholine added to the bath in different preparations, it was found that the response to antitoxin was 184, that to α-toxin 110 and that to the mixture 177. These were mean values from three experiments. Nevertheless there was an antagonistic effect of the antitoxin. It became evident after washing out the bath fluid. The contracture produced by the α-toxin was more or less irreversible, and the preparation was insensitive to further administration of α-toxin or of acetylcholine. On the other hand, the contracture produced by the antitoxin alone or by the antitoxin incubated with the α-toxin was reversible and the preparation responded to subsequent administrations of α-toxin or of acetylcholine.

The same results were obtained on the rabbit intestine. When a mixture of 10 H.U. of α-toxin and 25 units of antitoxin after incubation for 45 min. at 37° was tested on rabbit jejunum, it caused a contracture greater than that of the antitoxin alone (see Fig. 3 at **X** and **A** respectively). This greater effect did not appear to result from an action of the α-toxin itself since the contracture was fully reversible and the preparation remained sensitive to acetylcholine and to α-toxin which produced an irreversible contracture (at **T**). The greater effect of the mixture can easily be accounted for by the non-specific stimulating effect of the uninoculated culture medium which is shown in Fig. 1*a* at **C1** and **C2**. In fact, if the antitoxin was mixed with uninoculated culture medium, such a mixture produced a larger contracture than that following either alone.

If 50 to 100 units of antitoxin were added to the fluid bathing guinea-pig or rabbit preparations, and allowed to remain in contact with the intestine for 10 min., subsequent addition of α-toxin caused no increase in contracture. After replacing the bath fluid with fresh solution, the intestine responded to acetylcholine and to α-toxin.

When similar doses of antitoxin were added during a contracture in response to α-toxin, the contracture was not reduced and, when the preparation was washed, the contracture remained and the preparation no longer responded to further doses of α-toxin or to acetylcholine. This shows that, once the α-toxin has acted on the preparation, the antitoxin cannot reverse its action.

Relation of Haemolytic and Gut-contracting Activities

In the guinea-pig ileum and rabbit jejunum, 150 to 200 H.U. of purified α-toxin were required to produce a contracture of the same magnitude as 5 H.U. of the Wellcome α-toxin. The nature of the contracture produced by the two toxin samples was similar in that it was not reversed by washing but was opposed by papaverine and mepyramine. The greater stimulant activity/H.U. shown by the Wellcome toxin might have been due to its higher content of impurities derived from the culture medium. To test this possibility, experiments were carried out on the guinea-pig and rabbit intestine comparing the activity of the uninoculated culture medium and the Wellcome α-toxin. Strictly quantitative comparisons could not be made because of the uncertainty regarding

the amount of culture medium present in the Wellcome toxin.

With the guinea-pig ileum, addition of a quantity of uninoculated medium (approximately equivalent to that contained in 20 H.U. α-toxin) produced a much smaller effect than 20 H.U. α-toxin, although similar in time course (Fig. 2); with the rabbit jejunum the difference was even greater (Fig. 1*a* at **C1** and at **T**). In addition, in both preparations the response to the culture medium was readily reversible by washing and the sensitivity of the gut to acetylcholine and α-toxin remained undiminished. In both tissues a dose of atropine, sufficient to abolish the response to a dose of acetylcholine previously producing maximal stimulation, also abolished the effect produced by the culture medium while not affecting the contracture produced by the α-toxin (Fig. 2). Thus the uninoculated medium had not only a weaker action on the intestine, but its action was different from that of the Wellcome α-toxin samples. Therefore the constituents of this medium cannot wholly account for the activity of the impure sample.

From these results, it would appear that the gut-contracting and haemolytic properties of the α-toxin are due to different although perhaps related substances. Otherwise we would have to assume that the impure toxin contains a factor which facilitates the stimulant action of the α-toxin yet is itself inactive. If this were the case the factor would be thermolabile since addition of boiled Wellcome toxin to the purified toxin did not increase the activity of the latter.

Discussion

The contractures of rabbit jejunum and guinea-pig ileum following α-toxin resemble each other, being characterized by (a) a latent period between application of α-toxin and onset of contractures; (b) a slow rise to a maximum over a period of up to several minutes; (c) the persistent nature of the contractures; (d) the desensitization of the gut to further doses of α-toxin and to acetylcholine.

Feldberg and Kellaway (1938) described a contracture of the guinea-pig ileum to a staphylococcal toxin prepared by the method of Burnet and Freeman (1932). They also found that after a single dose the preparation was insensitive to further doses of the toxin. However, their contracture differed in that it developed more rapidly and was readily reversed by washing. The reversibility might be related to the fact that their toxin produced a contracture with a much shorter time of contact, and the differences in the methods of preparation of the toxins might be responsible for these discrepancies. On the other hand, Dworetzky, Baldwin, and Smart (1956) have since described a slow, delayed, irreversible contracture of guinea-pig ileum in response to 0.18 mg. of a dialysed culture filtrate of a pathogenic strain (Wood-46) of *S. aureus*. This finding is more in accord with our observations.

The α-toxin might have two actions, an early stimulant action followed later by a "toxic" action, the development of irreversibility and desensitization. This view is in accord with the findings that the decline in the response of the gut to acetylcholine following application of α-toxin depended on the time elapsed since addition of α-toxin. Also the observation that the prolonged presence in the bath of a spasmolytic, for instance papaverine, prevented the intestine from developing a contracture after the papaverine and the α-toxin were washed out could be explained on a similar basis.

As it has not been possible to separate the stimulant and the "toxic" activities by heat inactivation or antitoxin neutralization, we consider that, if they are not identical, then they must be closely associated and the term "gut-stimulant activity" will be used to cover both of the above hypothetical activities.

In the crude toxin, the ratio of gut-stimulant to α-haemolytic activity was about 40-fold greater than in the purified toxin. As the impurities in the crude toxin contributed but little to the height of the contracture and had effects which were readily reversible, the gut-stimulant and α-haemolytic activities may not be properties of the same principle although evidence of an association of these two activities with different principles in the α-toxin has yet to be forthcoming.

In this connexion, the following observations are of interest. The *in vivo* enterotoxic action, namely that action associated with staphylococcal food-poisoning in man, has been associated with *in vitro* gut-stimulant action on mammalian intestine by Richmond *et al.* (1942) and hence with the α-haemolytic fraction. However, Surgalla and Hite (1945) reported that heating culture filtrates to 100° for 30 to 40 min. did not abolish enterotoxic properties although destroying α- and β-haemolysins. More recently, on the other hand, Dworetzky *et al.* (1956) have claimed that heating to 100° for 15 min. or pre-treatment for 4 to 6 weeks with 0.3% formalin destroyed enterotoxic, *in vitro* gut-stimulant and

α-haemolytic properties of staphylococcal culture filtrates. At present, therefore, the physical relationship between these three properties and such principles in the culture filtrate with which they might be associated is still obscure.

Feldberg and Kellaway (1938) suggested that the stimulant action of staphylococcal toxin on gut is an indirect effect due to release of some stimulant factor. Such an indirect action could account for the latent period and slow rise of the toxin contracture. Feldberg and Keogh (1937) demonstrated the release of histamine by staphylococcal toxin from perfused guinea-pig lung, and Feldberg and Kellaway (1938) showed a similar release of histamine and another stimulant substance producing a delayed contracture of guinea-pig jejunum from perfused dog lung and liver. We have been unable to demonstrate the release of a contracture-producing substance from guinea-pig ileal loops by α-toxin, and a direct stimulating action on the gut wall cannot therefore be excluded. The action could be either on the muscle or through the neuronal network in the intestinal wall. The failure of atropine, cocaine, or hexamethonium to reduce the α-toxin contracture does not suggest an action on the neuronal network. Until convincing evidence for an indirect mode of action of the α-toxin can be found, a direct muscle-stimulating effect seems to be the most likely mechanism of action.

North and Doery (1958) recently described a protective action of a fraction of Australian tiger snake venom against the lethal and dermonecrotic effects of staphylococcal α-toxin in mice. One sixth of the LD50 of the venom fraction protected mice against a lethal dose of α-toxin when administered intravenously simultaneously with or up to 10 min. before the α-toxin, but not if injected after the α-toxin administration. A similar protection was found in the case of the dermonecrotic action of α-toxin. The authors suggested that the protective actions of the venom fractions may have been due to the prior attachment to the sites of action of the toxin. The finding that this protective action of snake venom does not occur if given after the toxin is of interest with regard to the similar relationship observed in the present experiments between α-toxin and antitoxin. This does not, however, imply that the mechanisms underlying the protective action of the venom and of the antitoxin are the same. It would be interesting to know whether the tiger snake venom exerts a protective action against the stimulant activity of α-toxin on mammalian intestine as it does against the lethal effect of α-toxin in mice.

This study was commenced at the suggestion of Dr. E. J. K. Penikett. The authors are indebted to him for help and advice, to Dr. L. Butler for a purified α-toxin sample, to members of the staff of the Wellcome Research Laboratories for advice and for the uninoculated culture medium, and to Dr. W. Feldberg for much help in connexion with the preparation of the manuscript.

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STUDIES OF A PROTEOLYTIC ENZYME FROM *SCHISTOSOMA MANSONI*

BY

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The presence of a proteolytic enzyme has been demonstrated in ground-up preparations of *Schistosoma mansoni*. A twenty-fold purification has been achieved by ultracentrifugation at *p*H 3.0; the enzyme has an optimum *p*H of 3.9 and a marked substrate specificity for haemoglobin. No significant proteolysis was observed either with whole serum at *p*H values of 3.9, 6.0 or 8.0, or with isolated serum proteins at *p*H ranges between 2.5 and 7.7. The evidence is discussed that this enzyme may be located in the intestine of the schistosomes and that it is, at least in part, responsible for the supply of amino acids to the organisms.

Little is known about the protein metabolism of *Schistosoma mansoni*. Rogers (1940) reported that the black pigment present in the intestine of *S. mansoni* is probably haematin formed by the digestion of haemoglobin from the host. The parasite must obtain nutrient materials, including amino acids, from the blood of the host, and it is conceivable that there is a mechanism for the degradation of one or more blood proteins, following ingestion into the intestine of the parasite. Halawani, Hafez, Newsome, and Cooper (1949) observed that haemoglobin disappeared from the medium in which *S. mansoni* survived *in vitro*.

If it were known precisely what amino acids are required by *S. mansoni* and by what mechanism the parasite obtains them, an opportunity might become available for the development of schistosomicidal drugs. An initial approach to this problem is reported in this paper, in which the demonstration, partial purification and properties of a proteolytic enzyme from *S. mansoni* are described. The enzyme shows a marked specificity for haemoglobin (Timms and Bueding, 1958).

METHODS AND MATERIALS

Survival Studies.—Survival of schistosomes in a synthetic medium was studied by the use of procedures reported previously (Ross and Bueding, 1950). The synthetic medium used in the present investigation contained the following constituents in 100 ml.: NaCl, 550 mg.; KCl, 1.25 mg.; Na_2HPO_4, 2.9 mg.; $NaHCO_3$, 20 mg.; glucose, 200 mg.; $CaCl_2$, 13.5 mg.; $MgCl_2$, 13.5 mg.; glutathione, 10 mg.; choline chloride, 0.2 mg.; calcium pantothenate, 0.2 mg.; ascorbic acid, 0.1 mg.; pyridoxine, 0.1 mg.; inositol, 0.1 mg.; niacin, 0.05 mg.; riboflavin, 0.02 mg.; thiamine, 0.01 mg.; carotene, 0.01 mg.; biotin, 0.005 mg.; folic acid, 0.005 mg. Tris (hydroxymethyl) aminomethane was used as a buffer in a final concentration of 0.0025 M. Before use, the *p*H of the medium was adusted to 8.0 with dilute NaOH. The composition of this medium, which represents a modification of the synthetic medium used previously, was arrived at by systematic changes in the concentrations of individual constituents. With a supplement of amino acids (see below), the worms survived from 2 to 9 days (Table I). Even in the absence of amino acids the worms remained alive for 1 to 3 days, while in the medium used in earlier experiments the worms died within 12 to 18 hr. (Ross and Bueding, 1950). Addition of purified fractions of protogen to this medium resulted in a marked increase in the survival of schistosomes (Ross and Bueding, 1950). Since these observations were made, protogen has been obtained in pure form and identified as α-lipoic acid (Brockman, Stockstad, Patterson, Pierce, Macchi, and Day, 1952; Reed, Soper, Schnakenberg, Kern, Boaz, and Gunsalus, 1952). Pure samples of α-lipoic acid, kindly supplied by Dr. T. H. Jukes and by Dr. I. C. Gunsalus, did not prolong the survival of schistosomes in this synthetic medium. Therefore, the observed effects of protogen fractions must have been due to another, as yet unidentified, factor.

Amino acids were prepared in stock solutions adjusted to *p*H 8.0 with dilute NaOH. All amino acids used were the "natural" (−)-isomers. In earlier experiments, a mixture of amino acids was

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used; the final concentrations in 100 ml. of synthetic medium were: lysine, 4 mg.; alanine, 5 mg.; methionine, 3.5 mg.; threonine, 3.5 mg.; valine, 3.5 mg.; arginine, 3.25 mg.; leucine, 3.8 mg.; *iso*leucine, 2.6 mg.; phenylalanine, 1.3 mg.; tryptophane, 1.0 mg.; histidine, 0.6 mg. This mixture will be referred to as "the original amino acid solution." Subsequently, another amino acid mixture was used. On addition of this mixture to the synthetic medium, the final concentrations/100 ml. were: leucine, 11.7 mg.; lysine, 9.2 mg.; aspartic acid, 8.3 mg.; valine, 8.0 mg.; α-alanine, 7.5 mg.; histidine, 6.4 mg.; glutamic acid, 6.1 mg.; phenylalanine, 4.7 mg.; serine, 4.0 mg.; proline, 3.9 mg.; glycine, 3.5 mg.; arginine, 2.9 mg.; tyrosine, 2.7 mg.; threonine, 2.6 mg.; tryptophane, 1.3 mg.; methionine, 0.9 mg.; and *iso*leucine, 0.5 mg. This mixture will be referred to as the "globin amino acid solution" because the proportion of the individual amino acids in this mixture is the same as in globin. The amino acid analysis of human globin (performed by Dr. Lawrence Rosner, Laboratory of Vitamin Technology, Chicago, Illinois) yielded the following results (%): leucine, 13.0; lysine, 11.1; aspartic acid, 10.0; valine, 9.7; α-alanine, 9.1; histidine, 7.8; glutamic acid, 7.4; phenylalanine, 5.7; serine, 4.8; proline, 4.7; glycine, 4.3; arginine, 3.5; tyrosine, 3.3; threonine, 3.2; tryptophane, 1.6; methionine, 1.1; *iso*leucine, 0.6. When human globin was added to the synthetic medium, its final concentration was 75 mg./100 ml. This concentration was found to be optimal.

Proteolytic Activity.—Before the determination of proteolytic activity, adult schistosomes were dissected from the mesenteric and portal veins of mice infected 6 to 8 weeks previously with cercariae of *S. mansoni*. They were immediately placed in a chilled solution containing NaCl 1.3×10^{-1}M, KCl 2.8×10^{-3}M, $CaCl_2,2H_2O$ 3×10^{-4}M, $MgCl_2,6H_2O$ 5×10^{-3}M and buffered at *p*H 7.5 with the sodium salt of glycylglycine (final concentration 0.05M).

In the earlier experiments the schistosomes were transferred to a chilled all-glass homogenizer, together with 1.0 ml. of 0.9% saline for every 50 pairs of schistosomes, and ground up. The resulting suspension was stored in ice for a period not exceeding 2 hr. until required.

In later experiments, the enzyme was prepared from an acetone-dried powder of the schistosomes, which could be stored *in vacuo* at 0 to 3° for up to 2 weeks without loss of activity. In this manner, an enzyme of more constant potency could be prepared.

Protein concentrations of the enzyme preparations were determined by measuring the density of an appropriately diluted sample in 30% trichloracetic acid at 420 mμ in a Beckman spectrophotometer.

Preparation of Substrate.—The substrate used for the assay of proteolytic activity was bovine haemoglobin. 2 g. crystalline bovine haemoglobin was dissolved in 10 ml. of distilled water and dialysed. The concentration of haemoglobin in the dialysed solution was determined spectrophotometrically (Hawk, Oser, and Summerson, 1954), and was adjusted to 2.5% with a solution containing streptomycin, 150 mg., penicillin, 200 mg., and nystatin, 20 mg./l. After centrifugation at 3,000 rev./min. for 30 min. in a refrigerated centrifuge, the supernatant was removed and used as the substrate solution. It was stored in the frozen state.

Assay of Proteolytic Activity.—A modification of the method of Anson (1938) was used. 0.1 ml. of substrate solution was incubated with 0.1 ml. of 0.9% saline, 0.05 ml. homogenate, and 0.025 ml. 0.4 M-malate buffer (*p*H 3.8) at 37° for periods varying from 30 to 90 min. with mechanical shaking. Control experiments were always carried out in which the enzyme was incubated without substrate, and the substrate without enzyme. The reaction was stopped by the addition of 0.3 ml. of 5% trichloracetic acid, and the substrate was added to those tubes incubated with enzyme alone. The tubes were cooled in ice for 5 min. to allow precipitation of excess protein, and were then centrifuged at 3,000 rev./min. for 10 min. The supernatants were carefully transferred to chilled tubes, and aliquots were removed for amino acid determinations.

The amounts of amino acids liberated by the enzyme were determined in earlier experiments as their equivalent in tyrosine, using the reagent of Folin and Ciocalteu (1927), and in later experiments by the colorimetric ninhydrin method of Moore and Stein (1948) as modified by Cocking and Yemm (1954). With the ninhydrin method the results are expressed as μg. of free amino-nitrogen, calculated from a standard curve using alanine. Aliquots of the reaction mixtures were adjusted to *p*H 5.0 by the addition of a predetermined quantity of 0.5 N-NaOH before assay by the ninhydrin method.

Results

Synthetic Medium.—In an attempt to obtain a "balanced" amino acid mixture suitable for the survival of *S. mansoni in vitro*, addition of amino acids not present in the synthetic medium, as well as increases and decreases in the concentrations of individual amino acids, of groups of amino acids, or of the entire amino acid mixture, failed to prolong the survival of schistosomes. Since schistosomes degrade haemoglobin (Rogers, 1940; Halawani *et al.*, 1949), it is possible that they require only the amino acids from the digestion of globin. If this is so, the parasite should be adapted to utilize these amino acids in a medium containing the same proportions as in digested globin. Accordingly, an amino acid mixture with a qualitative and quantitative composition similar to that of globin was tested. Addition of this solution consistently resulted in a longer survival time than that in the original amino acid

solution (Table I) or of numerous modifications to the latter. Globin itself was less effective, but when globin was added to a medium containing the original amino acid solution, the worms survived for as long as in globin amino acid solution (Table I). These observations suggested that some of the nutritional requirements of schistosomes are met by the amino acids

TABLE I

EFFECT OF AMINO ACID MIXTURES AND OF GLOBIN ON THE MEAN SURVIVAL TIME OF *S. MANSONI* IN A SYNTHETIC MEDIUM

Means are calculated from 27 separate observations. Survival times in groups 2, 3, 4 and 5 were significantly longer than in Group 1 ($P<0.01$). There was no significant difference of survival times between Groups 4 and 2 or Groups 5 and 3.

Group	Medium	Mean ± S.E. (Days)
1	No amino acids	2·1 ± 0·17
2	Original amino acid solution	3·4 ± 0·22
3	Globin amino acid solution	6·3 ± 0·24
4	Globin	4·2 ± 0·22
5	Globin + original amino acid solution	6·4 ± 0·24

contained in globin and that these amino acids become available to the parasite within the host through the digestion of ingested red cells. This could be brought about by the action of one or several proteolytic enzymes present in the alimentary canal of the worms.

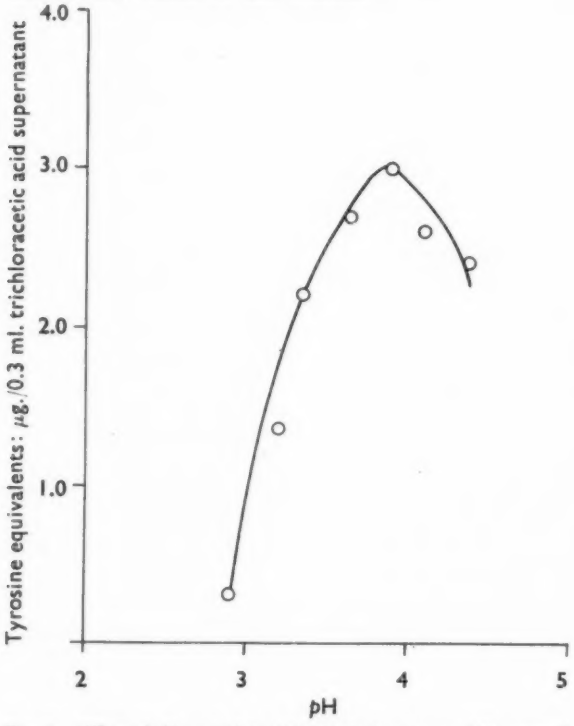

FIG. 1.—Effect of *p*H on activity of proteolytic enzyme of *S. mansoni*. 0·05 ml. of ground-up preparation was incubated for 45 min. at 37° with 1·0 mg. haemoglobin/ml.

Proteolytic Enzyme.—Preliminary experiments showed that homogenates of *S. mansoni* were able to degrade haemoglobin at *p*H ranges between 2.0 and 5.0 with an apparent optimum activity in the region of *p*H 4.0. A sample of the homogenate boiled for 3 min. showed no proteolysis at *p*H 4.0, indicating the enzymatic nature of the process. Direct proportionality was observed between the rate of degradation of haemoglobin and the concentration of enzyme, and also between the degree of degradation and the time of incubation with a fixed concentration of enzyme.

A more accurate determination of the *p*H optimum was made using a more dilute preparation (15 worms in 0.6 ml.), to avoid high blank readings in the colorimetric determinations. Phosphate buffers were used between *p*H 2.9 and 3.2 and malate buffers for other *p*H values. The final concentrations of the buffers in the incubation mixtures were approximately 0.04 M. The *p*H values stated are those of the final incubation mixture, as determined with a *p*H meter. Fig. 1 shows that the enzyme has a sharply defined optimum *p*H of 3.9.

The effect of substrate concentration is shown in Fig. 2. There is optimal activity at a

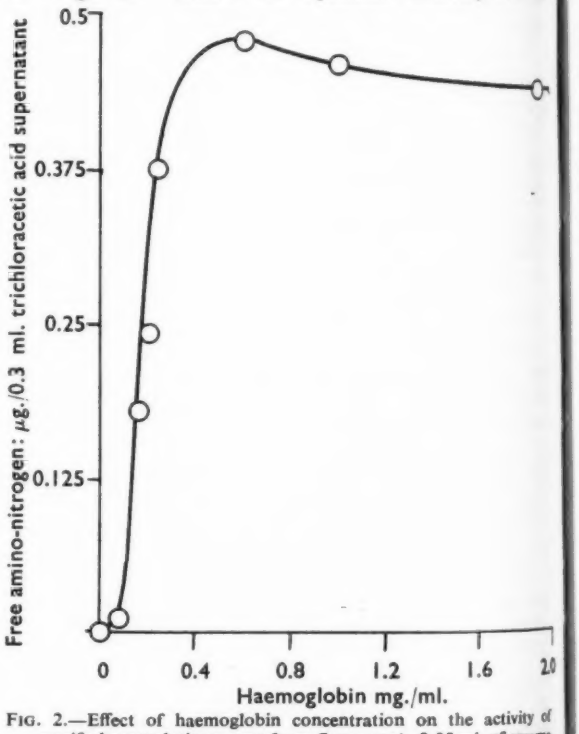

FIG. 2.—Effect of haemoglobin concentration on the activity of purified proteolytic enzyme from *S. mansoni*. 0·05 ml. of enzyme solution was incubated for 45 min. at 37° and *p*H 3·9 with 1 mg. haemoglobin/ml.

concentration of 0.5 mg. of haemoglobin/ml. incubation mixture. At 1.0 mg./ml., the activity is only slightly below the maximum; with concentrations of less than 0.5 mg./ml., the activity was reduced markedly.

Distribution of the Enzyme.—Both male and female schistosomes contained the enzyme. Separated male and female schistosomes were weighed, ground up in equal volumes of 0.45% saline, and assayed in duplicate at *p*H 3.9 with haemoglobin as the substrate. The female schistosomes contained 4.8 times the activity of the males/mg. of wet weight (Table II).

TABLE II
PROTEOLYTIC ACTIVITY OF MALE AND FEMALE SCHISTOSOMES

Activities expressed as μg. of free amino-nitrogen/0·3 ml. of trichloracetic acid supernatant released in 45 min. at 37° by 0·05 ml. of homogenates. Values are means of duplicate determinations.

	Wet Weight (mg.)	Activity	Activity/mg.	Ratio: Females/Males
Females ..	5·1	2·10	0·41	4·8
Males ..	9·4	0·80	0·085	

Purification of Enzyme.—An acetone-dried powder of freshly dissected schistosomes was found to possess 87.5% of the proteolytic activity found in the crude preparation, and to retain this for periods of up to 2 weeks *in vacuo* at 0 to 3°. The enzyme was found to be soluble and stable between *p*H 3.0 and 4.0. When a crude preparation of *S. mansoni* was adjusted to *p*H 3.8 and ultracentrifuged at 20,000 rev./min. (28,000 *g*) for 30 min., 45% of the initial activity was found in the clear supernatant. Under the same conditions, but in 0.02 M phosphate buffer at *p*H 3.0, 85% of the activity remained in the supernatant. When this procedure was applied to a suspension of an acetone-dried powder in 0.02 M phosphate buffer (*p*H 3.0), 82% of the original activity was recovered in the supernatant (Table III) though the protein concentration decreased to approximately one seventh of that in the suspension of acetone-dried powder. Therefore, the specific activity rose from 0.66 to 4.21.

TABLE III
PURIFICATION OF PROTEOLYTIC ENZYME FROM *S. MANSONI*

Activity expressed as μg. of free amino-nitrogen/0·3 ml. of trichloracetic acid supernatant. Incubated for 45 min. with 1·0 mg. of haemoglobin/ml. at *p*H 3·9 (37°). Specific activity was calculated as activity × 1,000/mg. protein/ml.

Step No.	Material	Activity	Protein (mg./ml.)	Specific Activity	Yield %
	Homogenate of schistosomes in 0·02 M phosphate (*p*H 3·0)	0·5	1·44	0·38	100·0
1	Acetone powder of schistosomes homogenized in 0·02 M phosphate (*p*H 3·0) ..	1·40	2·13	0·66	87·5
2	Supernatant after ultracentrifugation of 1. (20,000 rev./min. for 30 min.) ..	1·32	0·31	4·21	82·5
3	Supernatant after 2nd ultracentrifugation (39,000 rev./min. for 5 hr.)	1·06	0·13	7·80	66·5

Upon ultracentrifugation of the supernatant from Step 2 (see Table III) for 5 hr. at 39,000 rev./min. (108,000 *g*), 66.5% of the enzyme remained in the supernatant, although the protein concentration was less than half that in Step 2. The specific activity rose to 7.8, representing a twenty-fold purification compared with the crude preparation. The material from Step 3 will be referred to as "the purified enzyme." After partial purification the rate of amino-nitrogen production remained proportional to the enzyme concentration (Fig. 3) and the time of incubation (Fig. 4).

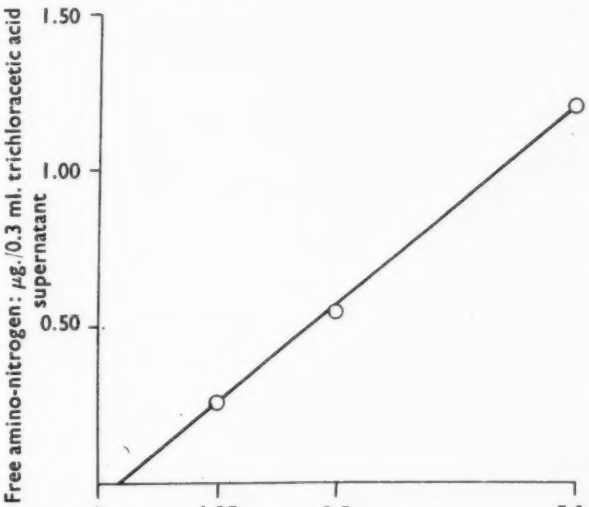

FIG. 3.—Relationship between concentration of proteolytic enzyme of *S. mansoni* and rate of haemoglobin degradation. Incubation was for 45 min. at 37° at *p*H 3·9 with 1·0 mg. of haemoglobin/ml.

Substrate Specificity of the Enzyme.—Table IV summarizes the results of substrate specificity studies using the purified enzyme. The enzyme showed a marked specificity for haemoglobin and globin, with both of which maximal proteolysis occurred at *p*H 3.9. No significant proteolysis was observed at any *p*H with any serum protein tested. A possible exception to this

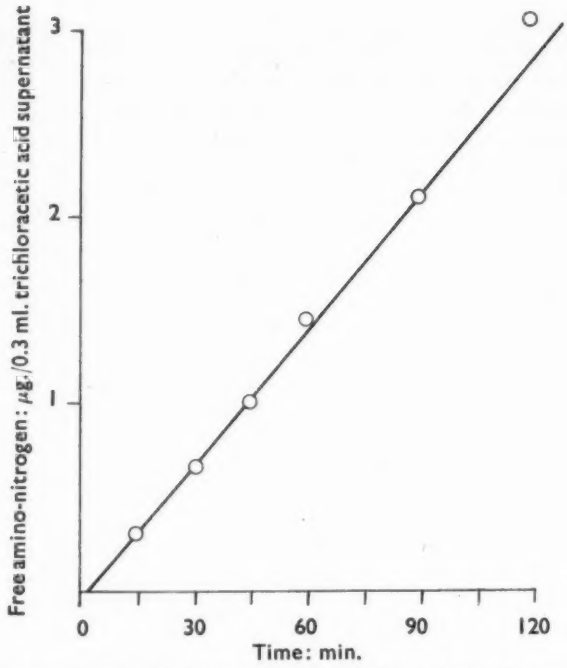

FIG. 4.—Relationship between time of incubation and rate of haemoglobin degradation. 0·05 ml. of homogenate was incubated at 37° and *p*H 3·9 with 1·0 mg. of haemoglobin/ml.

is bovine serum mercaptalbumen at *p*H 7.7. The last column of Table IV shows the amount of free amino-nitrogen released from haemoglobin by the enzyme under optimal conditions. These samples served as controls to ensure that the different batches of enzyme were active.

TABLE IV

SUBSTRATE SPECIFICITY OF PROTEOLYTIC ENZYME FROM *S. MANSONI*

The values are given as μg. of free amino-nitrogen/0·3 ml. of trichloracetic acid supernatant after 45 min. incubation at 37° with 0·05 ml. of purified enzyme.

*p*H	Substrate (1·0 mg./ml.)							
	Bovine Haemoglobin	Bovine Globin	Bovine Serum γ-Globulin	Porcine Serum γ-Globulin	Bovine Serum Albumen	Bovine Serum Mercaptalbumen	Bovine Serum Glycoprotein Fract. VI	Control Haemoglobin *p*H 3·9
2·50	0·0	0·10	0·0	0·10	0·0	0·0	0·0	1·50
3·90	1·70	1·35	0·13	0·15	0·0	0·13	0·0	1·70
5·50	0·39	0·50	0·0	0·01	0·0	0·0	0·0	1·20
6·60	0·0	0·0	0·0	0·01	0·20	0·0	0·25	1·50
7·70	0·60	0·10	0·0	0·0	0·0	0·50	0·0	2·00

Experiments were also made with two basic proteins, histone and protamine, and with lactoglobulin. These proteins could not be precipitated with trichloracetic acid in the normal manner, and gave very high blank readings if left in solution. The addition of 0.25 ml. of a 5% aqueous solution of zinc sulphate was found to precipitate these proteins; the amino acids remained in solution. None of these substrates was degraded by the enzyme at *p*H 3.9.

The reported survival of *S. mansoni* in undiluted horse serum (Robinson, 1956) raised the possibility that some serum protein, not tested above, may be degraded by the schistosomes. Accordingly, both the purified enzyme and the crude homogenate were tested for proteolytic activity using haemoglobin and dialysed human serum as substrates at *p*H 3.9, 6.0, and 8.0. Table V shows that free amino-nitrogen was produced

TABLE V

EFFECT OF PURIFIED ENZYME AND OF CRUDE HOMOGENATE OF *S. MANSONI* UPON DIALYSED SERUM

Activities are expressed as μg. of free amino-nitrogen/0·3 ml. of trichloracetic acid supernatant in 45 min. at 37°.

Substrate	Activity					
	Purified Enzyme			Crude Preparation		
	*p*H			*p*H		
	3·9	6·0	8·0	3·9	6·0	8·0
Haemoglobin	2·25	0·10	0·20	2·40	0·60	0·10
Human serum (dialysed)	0·00	0·30	0·35	0·30	0·00	0·00

from haemoglobin only at *p*H 3.9 (purified and crude enzymes), and at *p*H 6.0 (crude enzyme only). With neither enzyme was there a significant production of free amino-nitrogen from dialysed serum. The previously undetected degradation of haemoglobin at *p*H 6.0 by the crude preparation suggests the existence of a second proteolytic enzyme.

DISCUSSION

A solution containing amino acids in the same proportions as in globin increased the survival of *S. mansoni* in a synthetic medium to a greater extent than did other amino acid mixtures, and it therefore appears that the parasite is well adapted to utilize amino acids obtained from the digestion of haemoglobin. This process may represent a major source of essential amino acids for the worms; after the ingestion of red cells, the schistosomes deposit haematin crystals in their alimentary canals (Rogers, 1940), indicating that the intact worms digest haemoglobin. Furthermore, the activity of the proteolytic enzyme which degrades haemoglobin is approximately five times as high in the female as in the male worms (Table II). While the alimentary tract of both female and male schistosomes is of the same size, the males are three to five times as heavy as the females, due to the much greater weight of the musculature of

the males. Therefore, the higher proteolytic activity/mg. exhibited by preparations from female worms is consistent with the view that this enzyme is located in the alimentary canal. It remains to be determined whether the *p*H within the intestinal canal of the schistosome is at or near the optimum for the enzyme.

Undegraded globin was less effective in prolonging the survival of schistosomes *in vitro* than were the free amino acids contained in globin. Possibly, in the synthetic medium used, the activities of the proteolytic enzymes of the worms were lower than in the natural habitat of adult schistosomes. Addition of globin to the original synthetic medium prolonged survival; thus, even *in vitro*, some digestion of haemoglobin must have occurred. It is conceivable that in their natural habitat, schistosomes are able to utilize the free amino acids present in blood plasma as well as those produced by the digestion of haemoglobin.

The high degree of specificity of the proteolytic schistosome enzyme for haemoglobin and the inability of crude and purified preparations of the worms to hydrolyse any serum proteins to a significant degree (Table V) suggest that haemoglobin is the only host-protein which can supply schistosomes with amino acids. Therefore, interference with haemoglobin degradation by the worms might deprive the parasite of an essential source of amino acids and thereby provide an opportunity for the development of schistosomicidal agents. Furthermore, if, by the use of simple peptides of known structure, it can be established which bonds are hydrolysed by this proteolytic enzyme, the latter could serve as a tool for the elucidation of the amino acid sequence in the globin molecule.

After this paper had been submitted for publication, Cheever and Weller (1958) reported that addition of red cells, haemoglobin or globin to tissue culture media increased the survival of *S. mansoni in vitro*, while haemin had no effect. These findings provide additional support for our conclusions regarding the nutritional significance of the amino acids of globin for *S. mansoni*.

It is a pleasure to express appreciation to Mr. J. Hawkins and to Mr. H. Ruppender for able technical assistance. The authors are indebted to Dr. R. B. Pennell of Messrs. Sharp and Dohme Ltd. for supplies of human globin.

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RENAL EFFECTS OF VERATRIDINE

BY

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WITH AN APPENDIX BY P. W. TAILBY AND D. A. GILBERT

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Veratridine hydrochloride injected subcutaneously into unanaesthetized rats inhibited water diuresis. A linear relationship between log dose and antidiuretic effect could be established over the dose range 50 to 200 μg./100 g. of body weight. When veratridine hydrochloride was injected intravenously in doses from 10 to 30 μg./100 g., this relationship was also linear. In terms of its antidiuretic action, the alkaloid was approximately five times as effective when given intravenously. Rats anaesthetized with urethane responded to an intravenous injection with a more pronounced inhibition than unanaesthetized animals. Protoveratrine injected intravenously into unanaesthetized rats showed no clear relationship between dose and magnitude of antidiuretic effect. Veratridine hydrochloride injected intravenously had a pronounced hypotensive effect in both anaesthetized and unanaesthetized rats. Treatment with atropine did not affect this hypotensive action significantly. Atropine given subcutaneously 30 min. before an intravenous injection of veratridine hydrochloride abolished or diminished the inhibitory effect of veratridine on water diuresis. Veratridine hydrochloride injected intravenously into unanaesthetized rats caused a marked depression of the clearance of inulin and *p*-aminohippurate. In unanaesthetized rats with an osmotic diuresis, veratridine hydrochloride produced its usual antidiuretic effect. The urine of rats injected with veratridine hydrochloride produced an antidiuretic effect when injected intravenously into other animals. The antidiuretic potency of such urines was not affected by treatment with thioglycollate. Animals injected with veratridine excreted small amounts of a veratridine-like substance in the urine. These results do not suggest that veratridine in antidiuretic and hypotensive doses stimulated the neurohypophysis in the rat.

Ginsburg and Heller (1952, 1953a) and Ames and van Dyke (1952) found that blood obtained by heart puncture from rats contained more antidiuretic activity than blood taken from a carotid artery. The rats were anaesthetized, which made it unlikely that the neurohypophysis was stimulated by pain. Since, however, relatively large volumes of blood were rapidly withdrawn, stimulation of receptors in or near the heart is likely to have occurred.

It is known that, in certain mammalian species, nerve impulses originating from cardio-aortic receptor areas reach the central nervous system by way of the vagus. It has also been reported (Chang, Chia, Huang, and Lim, 1939 ; Andersson, 1951) that stimulation of the central end of a divided vagus causes liberation of posterior pituitary hormones. Brun, Knudson, and Raaschou (1945), Noble and Taylor (1953), Andersson and Larson (1954), and Smith (1957) have suggested that, following a fall in blood pressure, impulses arriving by way of such connexions stimulate the posterior pituitary, and Henry, Gauer, and Reeves (1956) have shown that stimulation of left atrial stretch receptors in the dog influenced water diuresis. It seemed of interest, therefore, to investigate whether stimulation of cardio-aortic baro- or stretch-receptors by veratrum alkaloids (Cerletti, Li, Alanis, and Aviado, 1951 ; Rothlin and Cerletti, 1954) produced a stimulation of the neurohypophysis. That this occurs is suggested by the work of Blackmore (1955), who found that the injection of protoveratrine into unanaesthetized dogs produced an inhibition of water diuresis which was not found in dogs with diabetes insipidus due to stalk section. In contrast are the results of Meilman (1952), who reported that the oliguria after the intravenous injection of protoveratrine into hypertensive patients was accompanied by a striking decrease in excretion of sodium and chloride in the urine. This would not be

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expected if the antidiuretic effect of protoveratrine were mainly due to the release of posterior pituitary hormones, since increased secretion of vasopressin and oxytocin would either leave the electrolyte output unchanged (Crutchfield and Wood, 1948; Barclay, Kenny, and Nutt, 1949; Chalmers, Lewis, and Pawan, 1951) or would possibly increase it (Dicker and Heller, 1946; Brooks and Pickford, 1957).

In view of the results of Ginsburg and Heller (1952, 1953) and Ames and van Dyke (1952) already mentioned, rats were used in the present investigation.

METHODS

Adult male albino rats of the Wistar strain were used. Those used for diuresis experiments weighed 160 to 230 g., and those for blood pressure experiments, 300 to 390 g.

Measurements of Blood Pressure. — With anaesthetized rats the method of Dekanski (1952) was used, but no dibenamine was given. With conscious rats the animal was anaesthetized with ether and short polythene cannulae were inserted into a carotid artery and a jugular vein. The rat was then given heparin and both cannulae were brought out through the skin at the back of the neck. When the rat regained consciousness extensions were fitted to the cannulae. The venous cannula was closed with a removable cap and the arterial cannula was connected with a manometer (Condon, 1951).

Tests for Antidiuretic Action.—The method of Heller and Zaidi (1957) was followed when test substances were injected intravenously, and that of Ginsburg (1951) when the test substances were injected subcutaneously; only two doses of water were given. The same animals were used again after an interval of two days, but were "crossed over."

Renal Clearance Estimations.—The method of Dicker and Heller (1945) was modified in that the bladder was cannulated on the day before the experiment. Immediately before and at the end of the clearance period the bladder was rinsed with tepid 0.9% NaCl solution. The animal was then anaesthetized with ether and blood was collected from a carotid artery. Ginsburg (1957) has shown that inulin clearances estimated in this manner are in good agreement with the results of experiments in which conscious rats received a constant intravenous infusion of inulin and in which several blood samples were taken. Inulin clearance (C_{IN}) and *p*-aminohippurate clearance ($C_{I\,AH}$) were determined simultaneously. Inulin was estimated according to the method of Schreiner (1950) and *p*-aminohippurate according to that of Smith, Finkelstein, Aliminosa, Crawford, and Graber (1945).

Inactivation Experiments with Thioglycollate.—The modification of the method of Ames and van Dyke (1951) due to Vogt (1953) was used.

Materials

Veratridine. — A stock solution was prepared from the pure substance supplied by Sandoz Laboratories, Basle. The base was converted into veratridine hydrochloride by adding a few drops of N-HCl. About four drops of the acid were needed to dissolve 2 mg. of the base. The solution was then neutralized with N-NaOH and made up to volume with 0.9% NaCl solution. The stock solution contained 20 mg. of veratridine hydrochloride/100 ml.

Puroverine. — Puroverine, a mixture of protoveratrine A and B, was supplied by Sandoz Laboratories, Basle. The stock solution contained 10 mg. of the preparation in 100 ml. 0.9% NaCl solution.

Other preparations used were: atropine sulphate (B.D.H.), Pularin (Evans), Pitressin (Parke, Davis), inulin (B.D.H.), *p*-aminohippuric acid (B.D.H.), and thioglycollic acid (B.D.H.).

RESULTS

The Antidiuretic Action of Subcutaneous Injections of Veratridine Hydrochloride in Unanaesthetized Rats

Fig. 1 shows the relationship between the dose of the alkaloid injected and its antidiuretic effect. The solutions of veratridine were dilute, neutral, and isotonic, but the rats resented the injection. They started to salivate profusely about 15 min. after the injection.

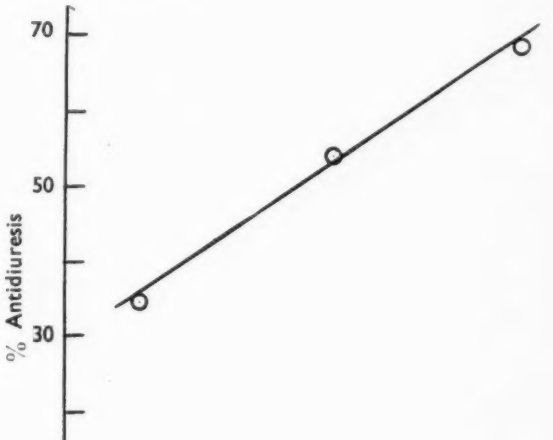

FIG. 1.—The antidiuretic action of veratridine hydrochloride injected subcutaneously in unanaesthetized male rats. Each point represents the mean of results in 12 animals.

The Antidiuretic Action of Intravenous Injections of Veratridine Hydrochloride

Fig. 2 shows the inhibitory effect of intravenous injections of the alkaloid on the water diuresis of conscious rats. The rats showed no resentment during the injection, but almost immediately after the injection had been completed the animals became restless; clonic convulsions occasionally occurred after large doses had been given. Gross changes in respiration were also noted in some instances. About 5 min. after the injection, the rats began to salivate profusely and continued to do so for about 10 min.

A comparison of Fig. 2 with Fig. 1 shows that the alkaloid was much more potent when injected intravenously. The antidiuretic effect of 20 μg./100 g. of body weight injected intravenously was approximately equal to the effect of 100 μg./100 g. injected subcutaneously.

Since unanaesthetized animals showed excitement shortly after the intravenous injection of veratridine and excitement itself is known to favour the release of antidiuretic hormone, the effect of intravenous injections of veratridine on water diuresis was also tested in anaesthetized animals. Pronounced antidiuretic effects were observed in rats anaesthetized with a 25% solution of urethane (0.7 ml./100 g.). The mean % antidiuresis in three such animals was 82.6 ± 7.2 (s.e.) which compares with 52.4 ± 4.2% (s.e., n=18) in conscious rats which had received the same dose (20 μg. of veratridine hydrochloride/100 g.). Thus veratridine was slightly more inhibitory in the anaesthetized rats ($P<0.001$).

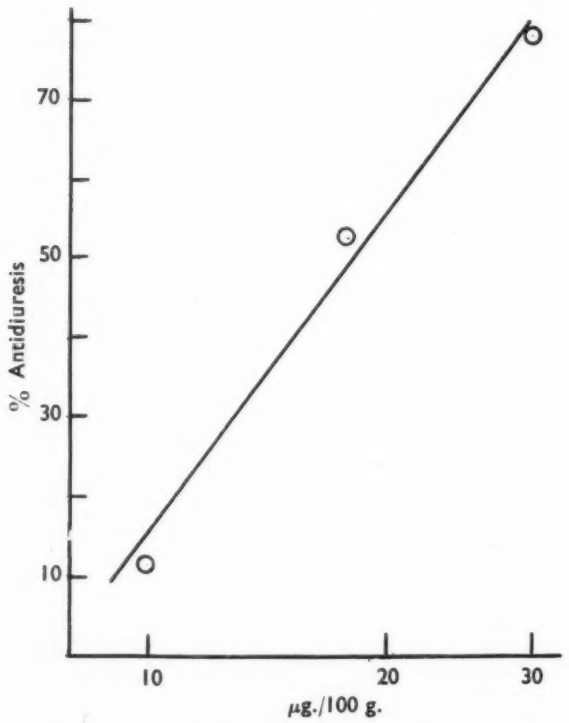

FIG. 2.—The antidiuretic action of veratridine hydrochloride injected intravenously in unanaesthetized male rats. Each point represents the mean of results in 6 to 18 animals.

Effect of Veratridine Hydrochloride on the Water Diuresis of Rats Treated with Atropine

Table I shows that atropine prevented the inhibitory effect of 10 μg. of veratridine hydrochloride/100 g. and that it diminished the antidiuretic effect produced by 20 μg. of veratridine/100 g. The antidiuretic effect of 30 μg. of veratridine was not much effected. Atropine itself in the dose and by the route given in these experiments had no effect on water diuresis: the mean % water load excreted in 3 hr. by 12 conscious rats which had been subcutaneously injected with 10 mg. atropine sulphate/100 g. was 63.5 ± 5.0 (s.e.) and that of 12 animals with control injections of 0.9% NaCl solution was 69.5 ± 5.6 ($P>0.4$).

TABLE I

EFFECT OF VERATRIDINE HYDROCHLORIDE ON THE WATER DIURESIS OF RATS AFTER ATROPINE

The animals were unanaesthetized; 10 mg. of atropine sulphate/100 g. was given subcutaneously 30 min. before veratridine was injected intravenously. Mean % antidiuresis is given with ± s.e. Number of animals used is given in parentheses.

Veratridine (μg./100 g.)	Veratridine Alone	Veratridine with Atropine	P
10	11·5±4·1 (6)	0·0 (6)	—
20	54·2±5·2 (15)	18·6±6·4 (15)	<0·001
30	78·2±4·1 (8)	59·6±8·6 (3)	>0·05

Effect of Puroverine Injected Intravenously on the Water Diuresis of Unanaesthetized Rats

Table II shows that the antidiuretic effect of puroverine was much less uniform than that of veratridine. There was less salivation, but the animals were much more excited and showed respiratory distress. Some animals injected with the highest dose of puroverine used (30 μg./100 g.) died in respiratory failure. The experiments with puroverine were therefore discontinued.

Effect of Veratrine Hydrochloride on the Systemic Blood Pressure of Rats

In view of the well-known hypotensive action of veratrum alkaloids in other mammalian species and in man, it seemed desirable to investigate

TABLE II

EFFECT OF INTRAVENOUS INJECTIONS OF PUROVERINE ON THE WATER DIURESIS OF UNANAESTHETIZED RATS

Animals marked with asterisk died shortly after conclusion of the experiment.

Animal No.	% Antidiuresis
7·5 μg./100 g.	
1	0
2	0
3	0
4	0
10 μg./100 g.	
1	0
2	7·5
3	80·5
4	0
15 μg. 100 g.	
1	0
2	52·5
3	0
4	9·4
5	0
6	84·0
7	5·0
8	11·2
20 μg./100 g.	
1	0
2	0
3	48·0
30 μg./100 g.	
1	23·3
2	60·7*
3	55·8*
4	44·6*
5	0
6	33·7
7	0
8	20·8
9	0

the effect of antidiuretic doses of veratridine on the blood pressure of the rat. Fig. 3*a* shows the marked depressor effect of 10 μg. of veratridine hydrochloride/100 g. injected intravenously in an unanaesthetized rat, and Fig. 3*b* shows that this effect was not inhibited by atropine. Hypotensive effects in rats anaesthetized with urethane were somewhat less pronounced and were hardly influenced by atropine. On some occasions veratridine injected after atropine gave a pure pressor response (Fig. 4).

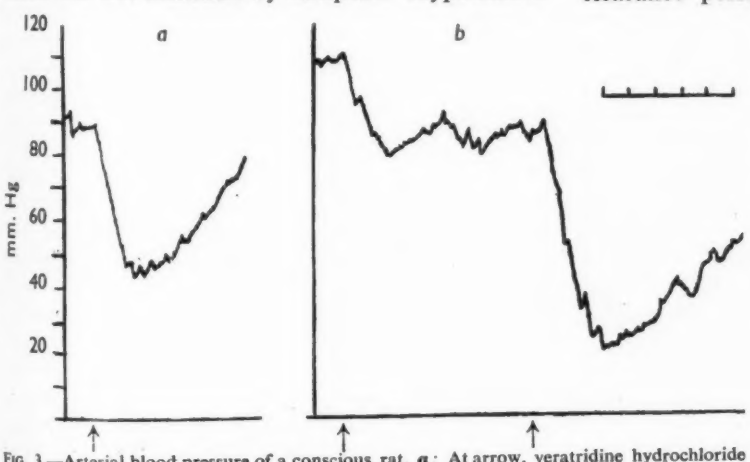

FIG. 3.—Arterial blood pressure of a conscious rat. *a*: At arrow, veratridine hydrochloride (10 μg./100 g.) intravenously. *b*: At first arrow, atropine sulphate (1 mg./100 g.) intravenously; at second arrow, veratridine hydrochloride (10 μg./100 g.) intravenously. Time, 1 min.

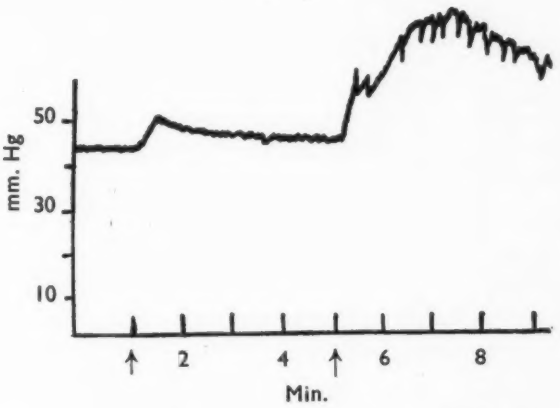

FIG. 4.—Pressor effect of veratridine: rat anaesthetized with urethane. Atropine sulphate (0·5 mg./100 g.) intravenously 10 and 5 min. before start of record. At first arrow, acetylcholine (1 μg./100 g.) intravenously; at second arrow, veratridine hydrochloride (20 μg./100 g.) intravenously.

Effect of Veratridine Hydrochloride on the Inulin and p-*Aminohippurate Clearance of Unanaesthetized Rats*

Since veratridine had a pronounced hypotensive effect in unanaesthetized rats (Fig. 3), it might be expected to modify glomerular filtration rate and renal blood flow. Inulin clearance (C_{IN}) was therefore estimated in conscious rats injected with 20 μg. of veratridine hydrochloride/100 g. The clearance period lasted 30 min. from the time when the intravenous injection was completed. C_{IN} (mean ± s.e.) in 12 such animals was 0.17 ± 0.026 ml. / 100 g. / min., and mean C_{IN} in 20 controls injected with 0.9% NaCl solution was 0.60 ± 0.037 ($P<0.001$). Simultaneous inulin and *p*-aminohippurate clearances (C_{PAH}) in another group of 7 rats injected with the same dose of veratridine gave the following mean results: C_{IN}, 0.17 ± 0.079, C_{PAH}, 0.49 ± 0.134. C_{IN} in 10 controls was 0.59 ± 0.035 and C_{PAH} 3.0 ± 0.452 ml. Thus veratridine lowered both clearances significantly ($P<0.001$).

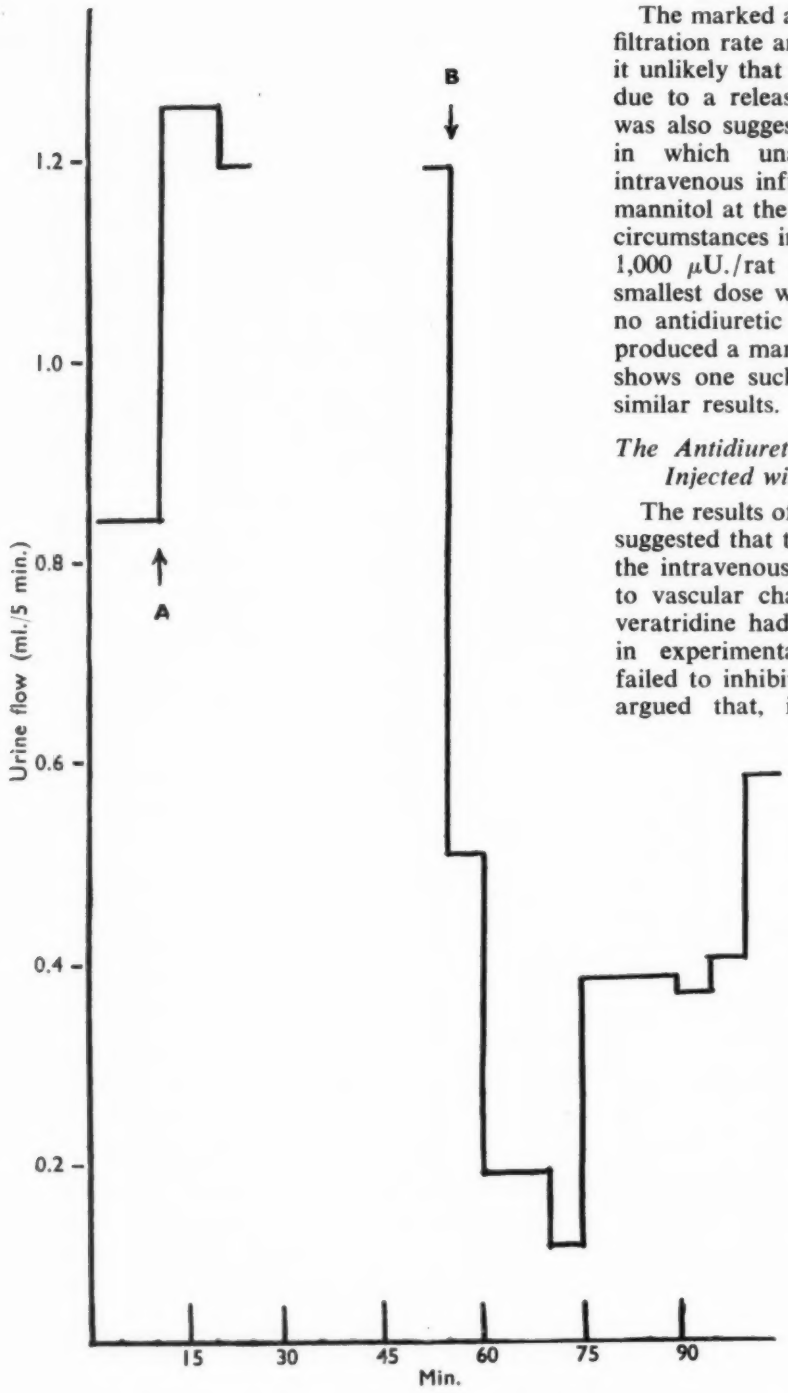

FIG. 5.—Antidiuretic effect of veratridine during osmotic diuresis. Unanaesthetized rat (315 g.) given intravenous infusion of 20% mannitol (0·1 ml./min.). A: pitressin (160 μU./100 g.) intravenously; B: veratridine hydrochloride (20 μg./100 g.) intravenously.

Effect of Veratridine Hydrochloride on Osmotic Diuresis

The marked action of veratridine on glomerular filtration rate and effective renal blood flow made it unlikely that the antidiuretic effects were solely due to a release of antidiuretic hormone. This was also suggested by the results of experiments in which unanaesthetized rats received an intravenous infusion of a solution of 20% (w/v) mannitol at the rate of 0.1 ml./min. Under these circumstances intravenous doses of pitressin up to 1,000 μU./rat (about 50 times as much as the smallest dose which inhibited water diuresis) had no antidiuretic effect. However, veratridine still produced a marked decrease in urine flow. Fig. 5 shows one such experiment. Three others gave similar results.

The Antidiuretic Activity in the Urine of Rats Injected with Veratridine Hydrochloride

The results of the clearance estimations strongly suggested that the antidiuretic effects produced by the intravenous injection of veratridine were due to vascular changes in the kidneys. Moreover, veratridine had a pronounced antidiuretic action in experimental circumstances when pitressin failed to inhibit urine flow. But it could still be argued that, in addition to having a direct antidiuretic action, veratridine caused the liberation of such large amounts of vasopressin that the renal vascular effect of this hormone became apparent. If this were so one would expect some of the released hormone to be excreted by the kidneys (Heller and Urban, 1935; Heller, 1952; Ginsburg and Heller, 1953b; Dicker, 1954; Heller and Zaidi, 1957). The urines of rats injected with veratridine were therefore tested for antidiuretic activity. The volume of urine voided 20 min. before an intravenous injection of 20 μg. veratridine hydrochloride/100 g. was measured and the urine excreted in the 20 min. after the injection was made up to the same volume with distilled water. Equal volumes of each urine were then injected into another rat. The urine collected after the injection of veratridine inhibited the water

diuresis of the recipient animal (Table III). However, Table III also shows that veratridine, added either to saline or to rat urine is not inactivated by thioglycollate. The antidiuretic substance excreted after an intravenous injection of veratridine was likewise not inactivated. The antidiuretic potency of such urines remained the same ($P>0.7$) before and after treatment with thioglycollate, suggesting that their inhibitory effect was solely due to the excretion of veratridine or some metabolic product of this alkaloid.

TABLE III

THE ACTION OF THIOGLYCOLLATE ON VERATRIDINE RESPONSE AND ON THE ANTIDIURETIC POTENCY OF URINE COLLECTED AFTER THE INTRAVENOUS INJECTION OF VERATRIDINE

All injections were made intravenously. Volume injected was 0·2 ml./100 g. throughout. Mean % antidiuresis is given with ± s.e. Number of animals used is given in parentheses.

Soln. No.		Mean % Antidiuresis
1	Urine collected for 20 min. *after* injection of veratridine hydrochloride (20 μg./100 g.) ..	39·9±6·1 (8)
2	Urine collected for 20 min. *before* injection of veratridine hydrochloride (20 μg./100 g.) ..	7·8±3·6 (8)
3	100 μg. of veratridine hydrochloride + 9 mg. of NaCl/ml. distilled water	61·5±6·3 (7)
4	As 3, but treated with thioglycollate ..	67·8±8·6 (7)
5	250 μU. of pitressin and 9 mg. NaCl/ml. distilled water	58·9±4·6 (7)
6	As 5, but treated with thioglycollate ..	8·1±3·8 (7)
7	Urine + 100 μg. added veratridine hydrochloride/ml.	78·8±6·3 (3)
8	As 7, but treated with thioglycollate ..	81·6±7·7 (3)
9	Urine + 250 μU. added pitressin/ml. ..	50·2±7·8 (3)
10	As 9, but treated with thioglycollate ..	13·6±6·8 (3)
11	Urine collected for 20 min. after i.v. injection of veratridine hydrochloride (20 μg./100 g.)	44·6±4·1 (6)
12	As 11, but treated with thioglycollate ..	42·8±4·5 (6)
13	Urine collected for 20 min. after the i.v. injection of pitressin (50 μU./100 g.) ..	37·4±5·8 (5)
14	As 13, but treated with thioglycollate ..	4·1±1·8 (5)

DISCUSSION

Fries, Stanton, Culbertson, Litter, Halperin, Burnett, and Wilkins (1949) investigated the oliguria caused by the intramuscular injection of veratrone (protoveratrine) into normotensive and hypertensive patients, and found that this preparation caused a fall in inulin and *p*-aminohippurate clearance. They noted, however, that the decrease in urinary volume persisted when renal plasma flow had returned to pre-injection values. During the period of depressed urine output, there was a marked rise of the inulin urine/plasma ratio. They stress that oliguria could also be produced by doses of protoveratrine which did not cause a significant reduction of systemic blood pressure. Likewise, Meilman (1953), who gave protoveratrine intravenously to similar groups of patients, emphasized that oliguria persisted in some patients after the glomerular filtration rate and renal plasma flow had returned to control values. During the period of oliguria, inulin urine/plasma ratios were increased and there was a striking diminution in the excretion of sodium and chloride.

The results of Nungesser and Hiatt (1954) in anaesthetized dogs injected with protoveratrine resemble, on the whole, those in man; the drug caused a decrease in urine volume with an increase of creatinine urine/plasma ratios which lasted longer than the decreases in blood pressure, glomerular filtration rate and renal plasma flow. The rise in inulin urine/plasma ratio, or in other words the increase in the tubular reabsorption of water, does not necessarily signify that the oliguria observed in these experiments was due to an increased release of antidiuretic hormone, since we know (del Greco and de Wardener, 1956; Berliner and Davidson, 1956) that a reduction of glomerular filtration rate may produce this effect by itself. However, the persistence of the oliguria for longer than the changes of blood pressure, glomerular filtration rate, and renal plasma flow is compatible with an antidiuretic effect due to the release of vasopressin. Blackmore (1955) injected protoveratrine into unanaesthetized dogs in doses which were probably not hypotensive (Maison, Gotz, and Stutzman, 1951) and obtained antidiuretic effects which were not seen when the same dose was administered to dogs with diabetes insipidus.

These findings can be compared with the results of the present investigation. It was possible to produce antidiuretic effects in rats with veratridine and protoveratrine, but the doses of protoveratrine needed to produce these effects were much higher than those required in the other species. Given by single intravenous injection, 3 μg. of protoveratrine/kg. reduced the urine flow in unanaesthetized dogs (Blackmore, 1955) and about 2 μg./kg. injected intravenously was needed in man (Meilman, 1953) whereas 300 μg. of the same alkaloid/kg. given by the same route to unanaesthetized rats failed to produce antidiuresis regularly. A similar comparison with veratridine cannot be made since this substance has apparently not been used to investigate renal effects in dogs and man. However, it could be shown that the inhibition of water diuresis in rats by veratridine resembles the oliguric effect of protoveratrine in other species in so far as a decrease in the clearances of inulin and *p*-aminohippurate was produced.

The blocking of parasympathetic effects by atropine had little or no effect on the fall of blood pressure produced by the intravenous injection of veratridine into rats, which is in accordance with the findings of Moe, Bassett, and Krayer (1944) in the dog. Occasionally a pure pressor response was seen in anaesthetized rats after atropine, which resembled that observed by Krayer and Acheson (1946) when large doses of veratridine had been given to dogs after vagotomy or atropinization. Atropine decreased the antidiuretic effect of small doses of veratridine but had little effect on the antidiuresis produced by large doses.

The marked effect of the intravenous injections of veratridine on the inulin and *p*-aminohippurate clearances made it unlikely that the antidiuretic effects produced in rats were due solely to stimulation of the neurohypophysis.

To establish whether veratridine releases vasopressin after the intravenous injection of antidiuretic doses in rats, it would have been desirable to investigate the effect of the alkaloid in neurohypophysectomized rats. However, since complete neurohypophysectomy in this species is notoriously difficult, another type of experiment was substituted. It is well known that the antidiuretic hormone ceases to depress urine flow during an osmotic diuresis (Adolph and Ericson, 1927; Melville, 1936; Paine and Nelson, 1940). Unanaesthetized rats were therefore given an infusion of 20% mannitol solution. In this condition, veratridine still produced its usual antidiuretic effect, whereas doses up to 1,000 μU. of pitressin had lost their inhibitory action. But it could still be argued that the amounts of vasopressin released by veratridine were so large (and it has been shown that very large amounts of vasopressin can be released by "noxious" stimuli from the rat pituitary (Ginsburg and Brown, 1956)) that the vascular effect of the hormone had contributed to the antidiuresis. However, if such large amounts were released, antidiuretic activity of neurohypophysial origin should appear in the urine (Heller and Urban, 1935; Heller, 1952; Ginsburg and Heller, 1953b; Dicker, 1954; Heller and Zaidi, 1957). Samples of urine excreted up to 20 min. after the intravenous injection of veratridine were therefore collected and injected into other rats in water diuresis. Such urines invariably had an antidiuretic action, but their antidiuretic activity was not inactivated by treatment with thioglycollate and the depression of urine flow did not differ significantly ($P>0.7$) before and after treatment with thioglycollate. It could also be shown by means of ultraviolet spectroscopy (see appendix) that after the injection of veratridine, the alkaloid or a chemically closely similar substance was excreted in the urine.

Assuming that veratridine did not suppress the renal excretion of vasopressin, and it will have been noted that it did not prevent its own excretion into the urine during the critical period, there is no evidence from these results in rats that veratridine caused a stimulation of the posterior pituitary. It may well be that veratridine differs in this respect from protoveratrine, but it is also possible that dogs and rats differ in their response to veratrum alkaloids for anatomical reasons. The afferent pathway for the aortic baroreceptor (and perhaps also coronary chemoreceptor) impulses (Dawes, 1947) appears to be peculiar in the rat (Andrew, 1954); and Blood, Kosman, and d'Amour (1955) have shown that, although pulmonary depressor reflexes can be elicited in rats, they are not abolished by vagal section as in other species. It would also seem that, in the rat, the receptors stimulated to release antidiuretic hormone by removal of blood following heart puncture are not identical with those that are activated by veratridine.

This work was part of that presented in a thesis by K. C. for the degree of Master of Science of the University of Bristol.

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APPENDIX

By P. W. TAILBY AND D. A. GILBERT

The antidiuretic substance excreted in the urine of rats after the intravenous injection of veratridine hydrochloride was investigated.

First, precipitation of the active substance was attempted by adding 2% phosphotungstic acid in 5% (v/v) H_2SO_4 to urine, and a colour reaction with concentrated sulphuric acid was sought. However, control experiments with urines to which veratridine hydrochloride had been added showed that concentrations of more than 500 μg. of veratridine/ml. urine were needed to give a positive result.

Next, extraction of veratridine from the urine samples was tried. By adding solid Na_2CO_3, the urine was brought to *p*H 10, extracted with a mixture of ether (3 vol.) and chloroform (1 vol.), and the extract shaken with 2N-HCl. A few drops of this acid extract were then put on a filter paper, dried in air, and sprayed with Dragendorff's reagent. When veratridine hydrochloride was added to rat urine, concentrations of 100 μg./ml. could be demonstrated by this method, but the results with the urines of rats which had been injected with small amounts of the alkaloid were negative. A more sensitive method had therefore to be adopted.

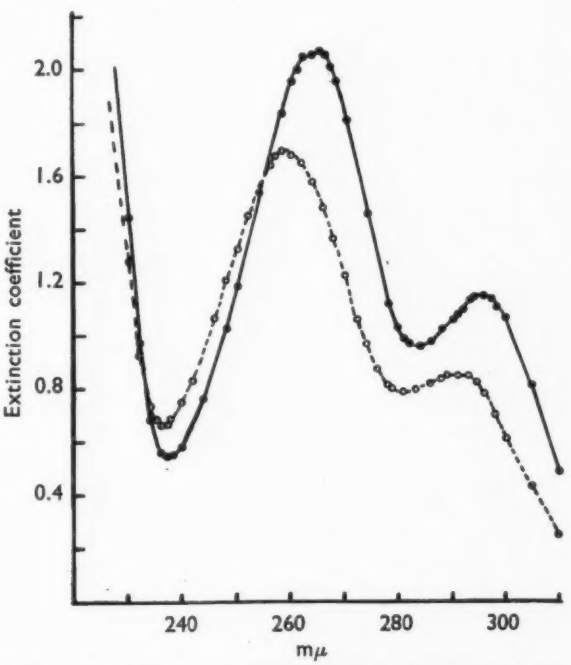

FIG. 1.—Ultraviolet absorption spectrum of veratridine hydrochloride dissolved in 0·9% NaCl solution. O - - - O: *p*H 5. ●——●: *p*H 3·6.

TABLE I

ESTIMATION BY ULTRAVIOLET SPECTROSCOPY OF THE VERATRIDINE-LIKE SUBSTANCE IN THE URINE OF A RAT INJECTED SUBCUTANEOUSLY WITH 100 μG. VERATRIDINE HYDROCHLORIDE

Urine which had been collected before the injection and to which 10 μg. veratridine/ml. had been added served as the standard. The collecting period in min. after the injection was given.

Collecting Period (Min.)	Urine Vol. Excreted (ml.)	Veratridine-like Substance Excreted (μg.)
0– 30	0·75	0·42
30– 60	0·40	1·40
60– 90	0·30	0·61
90–120	0·30	0·41

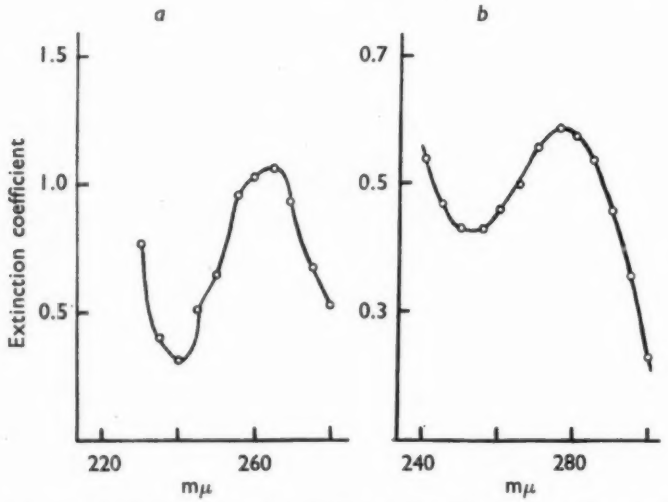

FIG. 2.—*a*: Ultraviolet absorption spectrum of veratridine hydrochloride added to rat urine. *b*: Spectrum of the urine of a conscious rat injected intravenously with veratridine hydrochloride (50 μg./100 g.). Urine was collected from a bladder cannula for 30 min. after the injection.

Preliminary experiments with solutions of pure veratridine hydrochloride showed that the alkaloid gave a characteristic absorption curve in the ultraviolet range (Fig. 1): there was a maximum at 265 mμ and the extinction coefficient at this value changed markedly (from $E^{1\,cm.}_{265} = 1.553$ to $E^{1\,cm.}_{265} = 2.077$) when the *p*H of the solution was altered from 5 to 3.6. Next, 50 μg. of veratridine hydrochloride/100 g. was injected subcutaneously into unanaesthetized rats (weighing about 200 g.) with bladders cannulated. Urine was collected for half an hour before and for 120 min. after the injection. The urine samples were then extracted with ether/chloroform as already described. Fig. 2 shows the ultraviolet absorption spectrum of these extracts. The spectrum of the urinary extract resembled that of pure veratridine very closely. In Table I the quantitative changes in the spectrogram show that the excretion of the veratridine-like substances reached a peak during the second 30 min. after the injection. The recovery of these substances in 2 hr. was 2.84% of the injected dose in terms of veratridine base. We are unable to say whether the urinary excretion of the veratridine-like factor proceeded beyond 2 hr. after the injection.

We are indebted to Professor E. W. Yemm for granting us facilities in his department.

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EFFECT OF VARIOUS ELECTROLYTES UPON CARDIAC AND SKELETAL MUSCULATURE

BY

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In rats kept on a low-potassium diet that contains only maintenance levels of magnesium, cardiac necroses and muscular cramps were readily induced by the oral administration of sodium perchlorate or disodium hydrogen phosphate. The precipitation of these cardiac and skeletal muscle changes by sodium chlorate was prevented by the prophylactic administration of either potassium or magnesium chlorides. The protective effect of these chlorides against the cardiotoxic and convulsive effects of disodium hydrogen phosphate has already been demonstrated by our earlier experiments. Sodium sulphate produced cardiac necroses in rats maintained on the same diet, and both potassium and magnesium chlorides had a prophylactic action. Unlike sodium perchlorate, however, sodium sulphate produced no muscular cramps under these conditions. Equimolecular amounts of sodium given in the form of sodium chloride (instead of sodium perchlorate, sodium sulphate, or disodium hydrogen phosphate) did not cause cardiac necroses or muscular cramps in rats maintained on the potassium-deficient diet. As the same three sodium salts, namely the perchlorate, the sulphate, and the hydrogen phosphate, produced cardiac necroses in rats sensitized by either a potassium-deficient diet or by certain corticoids, it seems that the anion must play a decisive rôle, since equivalent amounts of NaCl are ineffective.

Our investigations on the electrolyte-steroid-cardiopathy which is characterized by massive myocardial necroses (referred to subsequently as the cardiopathy) revealed that, in the corticoid-conditioned rat, the toxicity of sodium can be decisively influenced both by the anion with which sodium is associated and by the concurrent administration of various other cations. For example, in rats suitably conditioned with certain corticoids, it is possible to produce extensive myocardial necroses and fatal cardiac failure with Na_2HPO_4, NaH_2PO_4, $NaClO_4$, or Na_2SO_4, while equimolecular amounts of NaCl are ineffective. It has also been observed that the cardiopathy thus elicited by corticoids with sensitizing sodium salts is completely prevented by the simultaneous administration of $MgCl_2$ or KCl (Selye, 1958).

The importance of potassium and magnesium for the maintenance of normal cardiac structure is further substantiated by the numerous observations that rats kept on potassium-deficient (Follis, 1942; Schrader, Puckett, and Salmon, 1937; Thomas, Mylon, and Winternitz, 1940) or magnesium-deficient (Greenberg, Anderson, and Tufts, 1936; Lowenhaupt, Schulman, and Greenberg, 1950; Schrader *et al.*, 1937) diets tend to develop focal myocardial necroses. In magnesium-deficient animals, these lesions are frequently accompanied by convulsions. Subsequent studies revealed that, in rats maintained on a low-potassium diet that contained only maintenance levels of magnesium cardiac necroses developed, but the necroses could be prevented by the administration of KCl and $MgCl_2$. On the other hand, before this diet in itself produced any obvious morbid changes, Na_2HPO_4 supplements (unlike equivalent amounts of NaCl) rapidly provoke the development of severe cardiac necroses and muscular cramps; but even these can be prevented by KCl or $MgCl_2$. Obviously, Mg^{++} and HPO_4'' are of importance in the development of the syndrome usually ascribed simply to potassium deficiency, and, under certain conditions, magnesium and potassium can substitute for each other (Selye and Bajusz, 1958).

$NaClO_4$ and Na_2SO_4, like Na_2HPO_4, were highly effective in producing a cardiopathy in corticoid-conditioned rats. Hence, it seemed of interest to determine whether the perchlorate and dibasic sulphate of sodium would also be especially

toxic to animals maintained on a potassium-deficient diet and, if so, whether their toxicity could be counteracted by KCl and $MgCl_2$.

Materials and Methods

Ninety female Sprague-Dawley rats, with an initial mean body-weight of 50 g. (range, 46 to 56 g.), were placed on the low-potassium diet of the Nutritional Biochemicals Corporation (Cleveland, Ohio) for 8 days. This diet consists of: maize starch 64.2%; casein 30%; butterfat 3.5%; $CaCO_3$ 1.3%; NaCl 1%; and a vitamin mixture which provided in each 100 lb. of diet: vitamin A concentrate (200,000 units/g.) 4.5 g.; vitamin D concentrate (400,000 units/g.) 0.25 g.; α-tocopherol 5.0 g.; ascorbic acid 45.0 g.; inositol 5.0 g.; choline chloride 75.0 g.; menaphthone 2.25 g.; *p*-aminobenzoic acid 5.0 g.; nicotinic acid 4.5 g.; riboflavin 1.0 g.; pyridoxine hydrochloride 1.0 g.; aneurine hydrochloride 1.0 g.; calcium pantothenate 3.0 g.; biotin 20.0 g.; folic acid 90.0 mg.; vitamin B_{12} 1.35 mg. On the 9th day, the rats were subdivided into 9 equal groups and treated as indicated in Table I. $NaClO_4.H_2O$ (Fisher), KCl (Fisher), $MgCl_2.6H_2O$ (Merck), Na_2SO_4 (Merck), and Na_2HPO_4 (Fisher), all "Analytical Reagent" degree of purity, were administered at the dose of 0.5 m.mole, and NaCl (Brickman) at 1 m.mole, in 2 ml. of water, at 10 a.m. and 4 p.m., by stomach tube. In the groups which received two of these salts simultaneously, 0.5 m.mole of each was administered conjointly in 2 ml. of water, in order to keep the fluid volume constant.

Table I

Effect of various electrolytes upon cardiac and skeletal musculature

The 10 animals of each group were kept on the potassium-deficient diet described in the text. The means and standard errors of the necrosis and cramp scores are expressed in an arbitrary scale of 0 to 3.

Group	Treatment	Cardiac Necrosis	Muscular Cramps	Mortality (%)
I	None	0·1±0·1	0	0
II	$NaClO_4$	1·7±0·30	3·0±0	0
III	$NaClO_4$+KCl	0	0	0
IV	$NaClO_4$+$MgCl_2$	0·4±0·30	1·1±0·23	30
V	Na_2SO_4	1·1±0·40	0	0
VI	Na_2SO_4+KCl	0·1±0·1	0	0
VII	Na_2SO_4+$MgCl_2$	0·5±0·28	0	50
VIII	NaCl	0·1±0·1	0	0
IX	Na_2HPO_4	1·2±0·25	3·0±0	50

Muscular cramps were noted soon after the administration of $NaClO_4$ (Group II) and Na_2HPO_4 (Group IX), but the regular test for these cramps was performed one hour following the second gavage, that is, at 5 p.m. on the 9th day. The "Flick Test" was used for this purpose, because it proved to be highly effective in revealing a latent tendency for muscular cramps. The test consisted in placing the rat on a flat surface (such as a table) and giving it a flick on the sacral region with the index finger. Normal rats either showed no response to this slight irritation or took a few steps forward, while animals with a marked tendency to develop cramps (for instance, after heavy overdosage with $NaClO_4$) made spastic extensor movements with their hind paws. The results of this test were expressed in an arbitrary scale running from 0 to 3, in which 0 meant no spastic response; 1, brief extensor cramp which disappeared immediately; 2, prolonged extensor cramp; 3, spreading of the cramp to the whole body with a characteristic hunching of the back and extension caudally of the forelimbs. This peculiar muscular response was particularly characteristic of animals receiving $NaClO_4$. Occasionally, it also occurred after heavy overdosage with Na_2HPO_4, but the latter salt tended to cause a much more generalized tetanic state with tremor; this, incidentally, was similarly precipitated by the flick test. At none of the concentrations of Na_2SO_4 used did animals so treated give a response to the flick test.

If very large amounts of $NaClO_4$ or Na_2HPO_4 were given, muscular cramps occurred even in rats kept on normal diets. For the interpretation of our results it is important to note, therefore, that in a preliminary experiment on three groups of ten normal rats, similar to those described in Table I but kept on "Purina Fox Chow," the flick test was completely negative after treatment with comparable doses of $NaClO_4$, Na_2SO_4 and Na_2HPO_4, respectively.

The main experiment was terminated on the 10th day, that is, one day after the electrolyte treatment ceased. The hearts were fixed in neutral formalin and stained with the acid-fuchsin technique (Selye, 1958) for the demonstration of early prenecrotic changes, and with the silver nitrate technique of von Kossa for the histochemical detection of calcium. The intensity of the cardiac necroses was assessed in terms of an arbitrary scale of 0 to 3. The means of these readings, as well as those of the flick test, are listed (with standard errors) in Table I.

Results

Table I indicates that, following such a brief period on the potassium-deficient diet, almost no cardiac necrosis occurred in the control animals (Group I). On the other hand, a single day of treatment with either $NaClO_4$ (Group II), Na_2SO_4 (Group V) or Na_2HPO_4 (Group IX) sufficed to cause widespread cardiac necroses in the majority of the experimental animals. These necroses are virtually indistinguishable from those of the cardiopathy that have been described elsewhere (Selye, 1958). They were characterized by a widespread distribution, with definite predilection for the subendocardial layers of both ventricles and for the entire thickness of the right ventricle. In the initial stages, the affected muscle fibres stained deeply and selectively with our fuchsin technique, but later they were absorbed and replaced by histiocytes and connective tissue cells. At this

stage, individual fuchsinophilic fibres might still be seen in the immediate vicinity of the inflammatory foci. As shown by von Kossa's stain, the necrotic muscle fibres often, but not always, underwent calcification.

This cardiotoxic effect of $NaClO_4$ could be prevented or at least diminished by KCl (Group III) and $MgCl_2$ (Group IV). Na_2SO_4 was somewhat (but not significantly) less effective than $NaClO_4$ in producing these lesions; but here again KCl (Group VI) and to a lesser extent $MgCl_2$ (Group VII) effectively prevented the damage. Both the aggravation produced by $NaClO_4$ and Na_2SO_4, and the prevention of this by KCl and $MgCl_2$, were statistically highly significant in all instances ($P<0.01$). Only the prophylactic effect of $MgCl_2$ against damage by Na_2SO_4 was not statistically significant.

In view of the possible rôle played by sodium in the precipitation of this cardiac necrosis, it was interesting that NaCl proved totally ineffective in this respect. This was all the more significant as NaCl was given at a dose level of 2 m.mole, twice daily, so that the amount of sodium administered in this form was equivalent to that of the Na_2SO_4 and Na_2HPO_4 and to twice that of the $NaClO_4$; yet the three last-mentioned substances proved effective under the same circumstances.

The last group (IX), in which Na_2HPO_4 was given, merely served to confirm our earlier work on the toxicity of this electrolyte to rats maintained on our potassium-deficient diet, and we noted that, as expected, the salt induced severe cardiac necroses.

As to the production of the muscular cramps the results were very similar: $NaClO_4$ and Na_2HPO_4 produced intense muscular cramps when given alone. As with Na_2HPO_4 (Selye and Bajusz, 1958), this effect of $NaClO_4$ could be inhibited by KCl and to a lesser, but still highly significant, extent by $MgCl_2$. On the other hand, Na_2SO_4, like NaCl, elicited no neuromuscular disturbances.

In this short-term experiment, treatment with $NaClO_4$, Na_2SO_4 or NaCl during one day caused no mortality, while Na_2HPO_4 resulted in the death of half our animals. Among the chlorides prophylactically effective, KCl appeared to be preferable under these conditions, in that it not only inhibited the cardiac necroses and muscular cramps completely, but caused no mortality. $MgCl_2$ was not only somewhat less effective in regard to the prevention of cardiac and muscular lesions, but it induced a comparatively high mortality.

Discussion

The most interesting outcome of these investigations appears to us to be that the sodium salts ($NaClO_4$, Na_2SO_4, Na_2HPO_4) previously shown to produce cardiac necroses in rats which had received corticoids have the same effect in animals on a potassium-deficient diet. This similarity in the conditioning influence of potassium deficiency and of corticoid overdosage is emphasized by the fact that equivalent amounts of sodium given in the form of NaCl are ineffective in both these circumstances. Further, KCl and $MgCl_2$, which prevent the cardiac necroses produced by sensitizing sodium salts in corticoid-treated rats, exert this same protective effect in animals treated with sensitizing sodium salts while on a potassium-deficient diet. At least, in the case of the cardiopathy, the sensitization must be due to sodium, since all the salts of cations other than sodium consistently proved ineffective in producing cardiac necroses after corticoid treatment (Selye, 1958); yet the anion also plays a decisive rôle, since NaCl was ineffective, both after corticoid-conditioning and during potassium deficiency.

It is also interesting that in the experiments reported here, as in those concerning the cardiopathy (Selye, 1958), $MgCl_2$ can replace KCl as a prophylactic agent. The low-potassium diet which we used contained 52 mg./kg. of magnesium, which corresponded to the minimal amount necessary for growth according to Tufts and Greenberg (1938). However, even if we assume that our diet was simultaneously deficient in potassium and magnesium, it is difficult to understand why it should predispose to the production of cardiac necroses and muscular cramps by $NaClO_4$, Na_2SO_4, and Na_2HPO_4 but not by NaCl, and why the predisposition so induced should be prevented by either KCl or $MgCl_2$. It would be tempting to assume that the cardiac necroses are, in all instances, hypokalaemic. In this event, the sodium salts could act because of the well-known antagonism between sodium and potassium which has been demonstrated in many other test-objects. However, the pronounced hypokalaemia which is induced in rats by heavy overdosage with various mineralocorticoids produces neither cardiac necroses nor muscular cramps (Selye, 1958); indeed, in the monkey, the dog and man (but not the rat), such hypokalaemia is accompanied by flaccid muscular paralysis (Selye, 1950). It is also noteworthy that, in the present experimental series, even the animals which died with grade 3 cardiac necroses due to the Na_2SO_4 treatment

never showed any trace of muscular cramps; hence, the effect of sensitizing sodium salts upon cardiac and skeletal muscle cannot be inseparably interrelated. To this we may add that, in our previous studies on the effect of Na_2HPO_4 upon rats kept on this same potassium deficient diet, there developed, in addition to the cardiac necroses and muscular cramps, an intense nephrocalcinosis which was also readily prevented by either $MgCl_2$ or KCl (Selye and Bajusz, 1958). Apparently, in this respect magnesium can also substitute for potassium.

In view of all these findings, it is not possible to explain our observations by the over-simplified theory that the diet predisposes to the hypokalaemia, and that all the manifestations precipitated by the sensitizing sodium salts are merely the consequences of an aggravation of potassium deficiency by an excess of sodium. Further investigations will be necessary to elucidate the mechanism of these complex ionic interactions, but it is already evident that the conditioning effect of certain corticoids can be rather closely reproduced by an experimental dietary insufficiency.

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THE RELATIVE ACTIVITIES OF SOME TRYPTAMINE ANALOGUES ON THE ISOLATED RAT STOMACH STRIP PREPARATION

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The relative potencies of analogues of tryptamine and 5-hydroxytryptamine have been determined on the rat fundus preparation. This tissue had an amine oxidase activity, which, in the homogenate, was able to inactivate both tryptamine and 5-hydroxytryptamine to about the same degree. Amine oxidase inhibitors potentiated the action of tryptamine and many analogues on the isolated rat fundus preparation, but not the action of 5-hydroxytryptamine or of other hydroxytryptamines. This suggested that, in the isolated organ, the amine oxidase was unable to inactivate 5-hydroxytryptamine, but could inactivate tryptamine, 5-methoxytryptamine and many others. These results may be explained if it is supposed that tryptamine entered the cell, but because of the polar hydroxyl group 5-hydroxytryptamine did not. This hypothesis is supported by the oil/water partition coefficients. The structure/activity of the various tryptamine derivatives is discussed in the light of this assumption.

Variations in the structure of tryptamine and 5-hydroxytryptamine have mainly been made in a search for strong antagonists to 5-hydroxytryptamine. Much less work has been published on the relative potency of tryptamine derivatives as mimics of 5-hydroxytryptamine. Erspamer (1952a) and Freyburger, Graham, Rapport, Seay, Govier, Swoap, and Van der Brook (1952) studied the LD50 values of various substituted tryptamines. Erspamer (1952b, 1954) also tested analogues of tryptamine on the oestrous rat uterus preparation. Quadbeck and Röhm (1954) used the jejunum of the guinea-pig to study 5-methoxy- and 5-chloro-tryptamine, and Armstrong (1958) has tested tryptamine analogues for their ability to produce pain.

The rat stomach strip preparation (Vane, 1957) is extremely sensitive to 5-hydroxytryptamine-like compounds and has been used to study the relative potencies of substituted tryptamines, as they became available.

METHODS

The rat stomach strip was prepared as described by Vane (1957). It was mounted in a 5 ml. organ bath and bathed in Tyrode solution at 37°. Hyoscine hydrobromide (10^{-7}) was added to the Tyrode solution to minimize any acetylcholine-like effect and to reduce baseline irregularities. The movements of the muscle were recorded, not with a spring lever as originally described, but with a pendulum lever (Paton, 1957) of identical characteristics. The drugs were added to the organ bath in not more than 0.4 ml. saline, and the contraction of the stomach strip was allowed to develop for 90 sec. The bath was then washed out by overflow and the muscle stretched for 30 sec. The next dose of drug was added 2 or 3 min. later, giving a full cycle time of 4 or 5 min. In some experiments, the muscle was allowed to relax without additional stretching to obtain information on the duration of action of the compounds. A slow drip of Tyrode through the organ bath was maintained between each addition of drug. A vibrator mounted on the rim of the organ bath reduced friction effects between the writing point and the smoked paper.

The relative activities of the analogues of tryptamine are expressed in terms of molar potencies equiactive with 5-hydroxytryptamine (=1). For example, the contraction produced by a suitable dose of the test drug was bracketed between two doses of 5-hydroxytryptamine. This was repeated at a second concentration: from these values the number of nanograms of the test drug to give an equivalent contraction to 1 ng. 5-hydroxytryptamine was calculated. This relative activity was then recalculated as the number of moles of the test drug equiactive to one mole of 5-hydroxytryptamine. Thus, an

equipotent molar ratio of 100 means that 100 molecules of the test drug produce the same contraction as 1 molecule of 5-hydroxytryptamine. Most compounds were tested on at least two preparations. In some experiments, the relative potencies were determined both before and after the addition of an amine oxidase inhibitor to the bathing solution.

Spinal cats were prepared by the method of Kosterlitz, Krayer, and Matallana (1955). The blood pressure was recorded from a cannula in the left carotid artery with a mercury manometer.

Amine Oxidase Determination

The fundal portions of the stomachs from 24 rats were homogenized in 0.067 M sodium phosphate buffer of *p*H 7.4. The homogenate was dialysed against several changes of a large volume of phosphate buffer for some hours to reduce the normal respiratory activity. It was then diluted so that 1.5 ml. of the suspension contained the equivalent of 1 rat fundus. 1.5 ml. of the suspension was pipetted into each manometer flask. The centre well contained 0.2 ml. of 20% KOH solution dispersed over a piece of filter paper. The side arm contained 0.3 ml. of the substrate made up to give a final concentration when tipped of 0.0075 M. The gas space was filled with oxygen and the oxygen consumption of the preparation measured manometrically for 1 hr. One manometer was used as a thermobarometer and control readings were taken for flasks containing homogenate but no substrate. This procedure is an adaptation of the technique of Barlow, Blaschko, Himms, and Trendelenburg (1955).

Measurement of Dissociation Constants

All solutions were made up using distilled water, reboiled and cooled under a stream of nitrogen. 10 ml. of a 0.02 M solution of the amine was pipetted into a small tube and 1.0 ml. of 0.1N-HCl added to bring the *p*H down to below *p*H 2.0. The electrodes of a Pye *p*H meter, connected to an ink recorder, were lowered into the solution, together with two lengths of syringe needle tubing. Nitrogen was bubbled through one of these at a rate which gave adequate stirring and the other was connected to a micrometer syringe containing 5N-NaOH (carbonate free). The syringe was driven at a constant rate by a velodyne motor at a speed to deliver 0.02 ml. of alkali/min. As the *p*H of the solution was continuously recorded by the pen writer on moving paper, the titration curve was drawn directly, and the pK_a values were estimated from this curve.

Oil/Water Partition Coefficients

Olive oil B.P. was shaken with a buffer solution of *p*H 7.0 to remove any acidity. The emulsion was separated by centrifugation and the oil was washed in a similar manner with distilled water. Tryptamine hydrochloride and 5-hydroxytryptamine creatinine sulphate were dissolved in Tyrode solution. 100 ng. of 5-hydroxytryptamine base/ml., 4 μg. of tryptamine base/ml. and equal volumes of the amine solution and olive oil were shaken together for 15 min. The phases were separated and the aqueous phase assayed for 5-hydroxytryptamine or tryptamine content. An aliquot of the oil phase was then shaken for 30 min. with twice its volume of 0.3N-HCl to extract the amine from the oil. After separation, the aqueous phase was neutralized with 0.3N-NaOH and assayed for 5-hydroxytryptamine or for tryptamine. The concentration of amine in both water and oil was estimated in this way to eliminate the possibility of destruction or inactivation.

Materials

All drugs were added to the organ bath in a neutral saline solution. Most of the tryptamines were as hydrochlorides; the exceptions are designated in the acknowledgments. Throughout the paper, names for compounds have been used which indicate their relationship to the parent compound, rather than the more exact but less informative chemical name. The chemical structures and names used are all shown in Table II. Both iproniazid phosphate and phenyl-*iso*propylhydrazine hydrochloride were used as amine oxidase inhibitors. Where concentrations or doses are given, the tryptamines are expressed in terms of base. All other compounds are in terms of salt.

Results

All the amines tested caused contraction of the stomach strip. The relative activities varied from greater than 30,000 for indolemethylamine, which was the weakest, up to 1.5 for 5-hydroxy-α-methyltryptamine, one of the strongest. The log dose/response curves for the more active of the compounds were compared with that for 5-hydroxytryptamine and found to be parallel over the range of dose studied. Because of the high magnification of the lever and the length of the strip of tissue, the full dose/response curve was not examined. This may account for the differences in relative activities shown in this paper and that of Barlow and Khan (1959).

Another factor which may cause variation in the potency of these amines is the ability of amine oxidase in the tissue to limit the concentration of active compound at the receptor site. Using the analogy of potentiation of the activity of acetylcholine on smooth muscle by the inhibition of cholinesterase with neostigmine, the activity of the tryptamines was studied in the presence of amine oxidase inhibitors. With iproniazid (10^{-5}) or in later experiments with phenyl*iso*propyl-hydrazine (Horita, 1958) in the Tyrode, it was found that the action of many of the tryptamines was greatly potentiated. Fig. 1 illustrates this and shows that the potency of tryptamine can be increased more than forty times by an amine oxidase inhibitor. At the same time, it must be

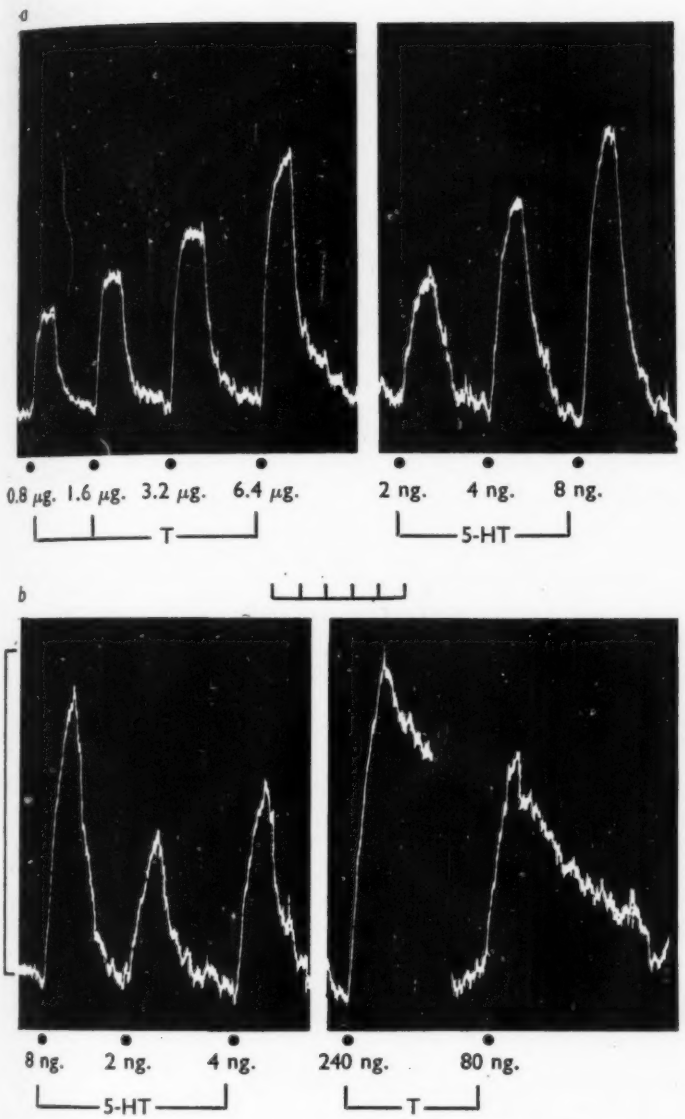


FIG. 1.—The response of the isolated rat stomach strip preparation to 5-hydroxytryptamine and tryptamine. (*a*) Responses to tryptamine (*T*) (Table II, No. 1) and 5-hydroxytryptamine (*5-HT*). (*b*) 45 min. after the addition of phenyl*iso*propylhydrazine (0·1 μg./ml.) to the Tyrode solution. The response to 5-hydroxytryptamine is unchanged, but that to tryptamine is potentiated, 80 ng. eliciting a contraction equivalent to more than 3·2 μg. before amine oxidase inhibition. Note also the slowness of relaxation after washing out the tryptamine compared to (*a*). Time, min. Vertical scale, 10 cm.

noted that the potency of 5-hydroxytryptamine was unaffected by inhibition of amine oxidase. This differential potentiation was always obtained. In some experiments there was a gradual increase in sensitivity to all drugs including 5-hydroxytryptamine, but the addition of an amine oxidase inhibitor did not noticeably accelerate this process for 5-hydroxytryptamine. In view of the well-substantiated fact that amine oxidase preparations studied by manometric techniques oxidize both tryptamine and 5-hydroxytryptamine at about the same rate (Blaschko, 1952; Freyberger *et al.*, 1952), this result was puzzling. To confirm the presence of an amine oxidase-like enzyme in the rat fundus, and to study its characteristics, rat fundi were finely ground up and the ability of this material to oxidize tyramine and some of the tryptamines was measured manometrically. The results are shown in Table I. It can be seen that an enzyme with the accepted characteristics of amine oxidase was present in the tissue. It oxidized tyramine at a fairly fast rate, and tryptamine, 5-hydroxytryptamine, and 5-methyltryptamine at about 60% of this rate. It had no action on 5-hydroxy-α-methyltryptamine and was completely inhibited by iproniazid (10^{-5}). The initial substrate concentration was 0.0075 M or between 1.0 and 1.5 mg./ml. for the amines used (as base). One fifth to one third of the total amount of amine was oxidized by the enzyme in 1 hr., a rate of about 6 μg./rat fundus/min. for tryptamine and 5-methyltryptamine and 7 μg./rat fundus/min. for 5-hydroxytryptamine. Thus, if the highly artificial conditions in a Warburg flask can be compared with those in an isolated organ bath, it is possible to say that the amount of amine oxidase present in a rat fundus was more than enough to account for the potentiation of the activity of tryptamine by amine oxidase inhibitors. The immunity of 5-hydroxytryptamine from attack by amine oxidase in the organ bath when it can be oxidized at about the same rate as tryptamine by the tissue homogenate is still unexplained.

The potentiation of the action of tryptamine, but not 5-hydroxytryptamine, on the stomach strip by the addition of amine oxidase inhibitors afforded a means of studying the characteristics of the enzyme in the whole tissue. Table II compares the equipotent molar ratios of the

TABLE I

THE OXIDATION OF TRYPTAMINE AND DERIVATIVES AND TYRAMINE BY A HOMOGENATE OF RAT FUNDUS

The respiration of the enzyme without substrate has been subtracted from all the values. The average value was 20 μl. O_2/g. of tissue/hr. The addition of iproniazid (10 μg./ml.) completely inhibited the oxidation of all the substrates.

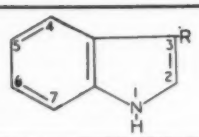
Substrate	O_2 Consumption (μl./g. of tissue/hr.)	Substrate (as Base) Oxidized, Calculated from Average Wet Weight of Fundus of 400 mg. (μg./rat fundus/min.)
Tyramine	207	8·4
5-Hydroxytryptamine ..	142	7·4
Tryptamine	124	5·9
5-Methyltryptamine	112	5·8
5-Hydroxy-α-methyltryptamine	0	0

tryptamines both before and after inhibition of amine oxidase. For the series of tryptamines unsubstituted in the indole ring, the potentiation of activity by inhibition of amine oxidase fitted well with the established ideas about the enzyme. For instance, the introduction of a methyl group on the α-carbon of the side-chain prevented oxidation by amine oxidase. Thus, when the enzyme was uninhibited, α-methyltryptamine (Table II, No. 4) has more than 10 times the potency of tryptamine (No. 1). However, after inhibition of the enzyme the contraction due to α-methyltryptamine remained the same height, but the potency of tryptamine increased, so that tryptamine was the more potent. Similarly, the introduction of two methyl, ethyl or propyl groups on the terminal amino group (Nos. 10, 11, and 12) apparently increased the potency, but that this increase in potency was only due to a decreasing action of amine oxidase was confirmed by repeating the experiments in the presence of iproniazid or phenyl*iso*propylhydrazine.

TABLE II

EQUIPOTENT MOLAR RATIOS OF TRYPTAMINE DERIVATIVES ESTIMATED ON THE ISOLATED RAT STOMACH STRIP, BEFORE AND AFTER AMINE OXIDASE INHIBITION

The numerals in brackets refer to the number of experiments on each compound. 5-Hydroxytryptamine was taken as unity.



No.	Ring Substituent	Side-chain R	Trivial Name Used in Text and Systematic Chemical Name	Normal (A) Equipotent Molar Ratio. Mean ± S.E. (No. of Tests)	After Amine Oxidase Inhibition (B) Equipotent Molar Ratio. Mean ± S.E. (No. of Tests)	Potentiation (A/B)
1	—	$-CH_2-CH_2-NH_2$	Tryptamine 3-(2-Aminoethyl)indole	408 ± 49 (15)	32 ± 4·8 (11)	12·7
2	—	$-CH_2-NH_2$	Indolemethylamine 3-Aminomethylindole	29,000 (2)	33,000 (2)	0·9
3	—	$-CH_2-CH_2-CH_2-NH_2$	Homotryptamine 3-(3-Aminopropyl)indole	1,920 (2)	460 (2)	4·2
4	—	$-CH_2-CH(CH_3)-NH_2$	α-Methyltryptamine 3-(2-Aminopropyl)indole	31 ± 5 7 (5)	40 (2)	0·8
5	—	$-CH_2-CH(C_2H_5)-NH_2$	α-Ethyltryptamine 3-(2-Aminobutyl)indole	4,600 (2)	—	—
6	—	$-CH_2-C(CH_3)_2-NH_2$	$\alpha\alpha$-Dimethyltryptamine 3-(2-Amino-2 : 2-dimethylethyl)indole	1,400 (1)	—	—
7	—	$-CH_2-CH_2-NH(CH_3)$	*N'*-Methyltryptamine 3-(2-Methylaminoethyl)indole	1,120 ± 192	126 (1)	8·9
8	—	$-CH_2-CH_2-NH(C_2H_5)$	*N'*-Ethyltryptamine 3-(2-Ethylaminoethyl)indole	250 (2)	170 (1)	1·5
9	—	$-CH_2-CH_2-NH(C_3H_7)$	*N'*-Propyltryptamine 3-(2-Propylaminoethyl)indole	330 (2)	330 (1)	1·0
10	—	$-CH_2-CH_2-N(CH_3)_2$	*N'N'*-Dimethyltryptamine 3-(2-Dimethylaminoethyl)indole	196 ± 30 (3)	110 (1)	1·8
11	—	$-CH_2-CH_2-N(C_2H_5)_2$	*N'N'*-Diethyltryptamine 3-(2-Diethylaminoethyl)indole	83 (2)	87 (1)	1·0
12	—	$-CH_2-CH_2-N(C_3H_7)_2$	*N'N'*-Dipropyltryptamine 3-(2-Dipropylaminoethyl)indole	34 (2)	41 (1)	0·8
13	2-CH_3	$-CH_2-CH_2-N(CH_3)_2$	2 : *N'N'*-Trimethyltryptamine 3-(2-Dimethylaminoethyl)-2-methylindole	1,200 (1)	—	—

TABLE II (*continued*)

14	5-OH	$-CH_2-CH_2-NH_2$	5-Hydroxytryptamine 3-(2-Aminoethyl)-5-hydroxyindole	1·0	1·0	1·0
15	4-OH	$-CH_2-CH_2-NH_2$	4-Hydroxytryptamine 3-(2-Aminoethyl)-4-hydroxyindole	1·8 (2)	2·0 (2)	0·9
16	6-OH	$-CH_2-CH_2-NH_2$	6-Hydroxytryptamine 3-(2-Aminoethyl)-6-hydroxyindole	460 (2)	560 (2)	0·8
17	$6-OCH_3$	$-CH_2-CH_2-NH_2$	6-Methoxytryptamine 3-(2-Aminoethyl)-6-methoxyindole	1,520 (2)	950 (2)	1·6
18	$5:6-(OCH_3)_2$	$-CH_2-CH_2-NH_2$	5 : 6-Dimethoxytryptamine 3-(2-Aminoethyl)-5 : 6-dimethoxyindole	300 (2)	147 (2)	2·0
19	$5-OCH_3$	$-CH_2-CH_2-NH_2$	5-Methoxytryptamine 3-(2-Aminoethyl)-5-methoxyindole	20±4 (4)	1·7±0·2 (4)	11·8
20	$5-OCH_3$	$-CH_2-CH(CH_3)-NH_2$	5-Methoxy-α-methyltryptamine 3-(2-Aminopropyl)-5-methoxyindole	2·9±0·6 (3)	2·5 (2)	1·2
21	5-Cl	$-CH_2-CH_2-NH_2$	5-Chlorotryptamine 3-(2-Aminoethyl)-5-chloroindole	100 (2)	7·6 (2)	13·2
22	$5-CH_3$	$-CH_2-CH_2-NH_2$	5-Methyltryptamine 3-(2-Aminoethyl)-5-methylindole	184±42 (3)	9 (1)	20·0
23	$5-CH_3$	$-CH_2-CH(CH_3)-NH_2$	5 : α-Dimethyltryptamine 3-(2-Aminopropyl)-5-methylindole	14±3·3 (3)	16 (1)	0·9
24	5-OH	$-CH_2-CH(CH_3)-NH_2$	5-Hydroxy-α-methyltryptamine 3-(2-Aminopropyl)-5-hydroxyindole	1·4±0·1 (7)	2·0 (2)	0·7
25	5-OH	$-CH_2-CH(C_2H_5)-NH_2$	α-Ethyl-5-hydroxytryptamine 3-(2-Aminobutyl)-5-hydroxyindole	7·0 (1)	—	—
26	5-OH	$-CH_2-CH_2-NH(CH_3)$	5-Hydroxy-*N'*-methyltryptamine 5-Hydroxy-3-(2-methylaminoethyl)indole	6·3 (2)	9·5 (1)	0·7
27	5-OH	$-CH_2-CH_2-N(CH_3)_2$	5-Hydroxy-*N'N'*-dimethyltryptamine 3-(2-Dimethylaminoethyl)-5-hydroxyindole	10±3·6 (3)	8·0 (1)	1·2
28	5-OH	$-CH_2-CH_2-N(C_2H_5)_2$	*N'N'*-Diethyl-5-hydroxytryptamine 3-(2-Diethylaminoethyl)-5-hydroxyindole	20 (1)	20 (1)	1·0
29	5-OH	$-CH_2-CH_2-N(C_3H_7)_2$	5-Hydroxy-*N'N'*-dipropyltryptamine 3-(2-Dipropylaminoethyl)-5-hydroxyindole	4·0 (2)	6·0 (2)	0·7
30	5-OH	$-CH_2-CO_2H$	5-Hydroxyindoleacetic acid 5-Hydroxyindol-3-ylacetic acid	Inactive	—	—
31	$5-OCH_3$	$-CH_2-CO_2H$	5-Methoxyindoleacetic acid 5-Methoxyindol-3-ylacetic acid	,,	—	—

For tryptamine derivatives with a hydroxyl group on the indole ring, the results were altogether different. Inhibition of amine oxidase did not potentiate the action of these compounds. An increase in the activity of 4-, 5-, and 6-hydroxytryptamine and the *N'*-substituted 5-hydroxytryptamines has never been observed after amine oxidase inhibition. On the other hand, the actions of methoxytryptamines (Fig. 2), 5-chlorotryptamine, and 5-methyltryptamine are all potentiated by amine oxidase inhibition (Table II, Nos. 17, 18, 19, 21, and 22).

The inability of the amine oxidase in the rat stomach preparation to act on the hydroxytryptamines suggested that a diffusion barrier existed which allowed tryptamine, but not 5-hydroxytryptamine, to pass. As the ability of this barrier to distinguish between tryptamine and 5-hydroxytryptamine might depend upon the physicochemical properties of the compounds, the

TABLE III

*p*K VALUES AND OIL/WATER SOLUBILITY COEFFICIENTS

The estimated *p*K values are assignable as follows: pKa_1, creatinine; pKa_2, terminal amino group; and pKa_3, ring hydroxyl group. The partition coefficient (concentration in oil/concentration in water) is given at the lowest initial concentration in the Tyrode solution to give easily assayable values. (100 ng. of 5-hydroxytryptamine base/ml. and 4 μg. of tryptamine base/ml.). The total recovery from both phases was 95% for 5-hydroxytryptamine and 116% for tryptamine.

No.		pKa_1	pKa_2	pKa_3	Partition Coefficient
1	Tryptamine hydrochloride	—	10·2	—	0·92
14	5-Hydroxytryptamine creatinine sulphate ..	4·9	10·0	11·1	0·055
27	5-Hydroxy-*N'N'*-dimethyltryptamine hydrochloride	—	9·8	11·2	
20	5-Methoxy-α-methyltryptamine hydrochloride	—	10·3	—	
12	*N'N'*-Dipropyltryptamine hydrochloride	—	8·6	—	
	Creatinine hydrochloride ..	4·9	—	—	

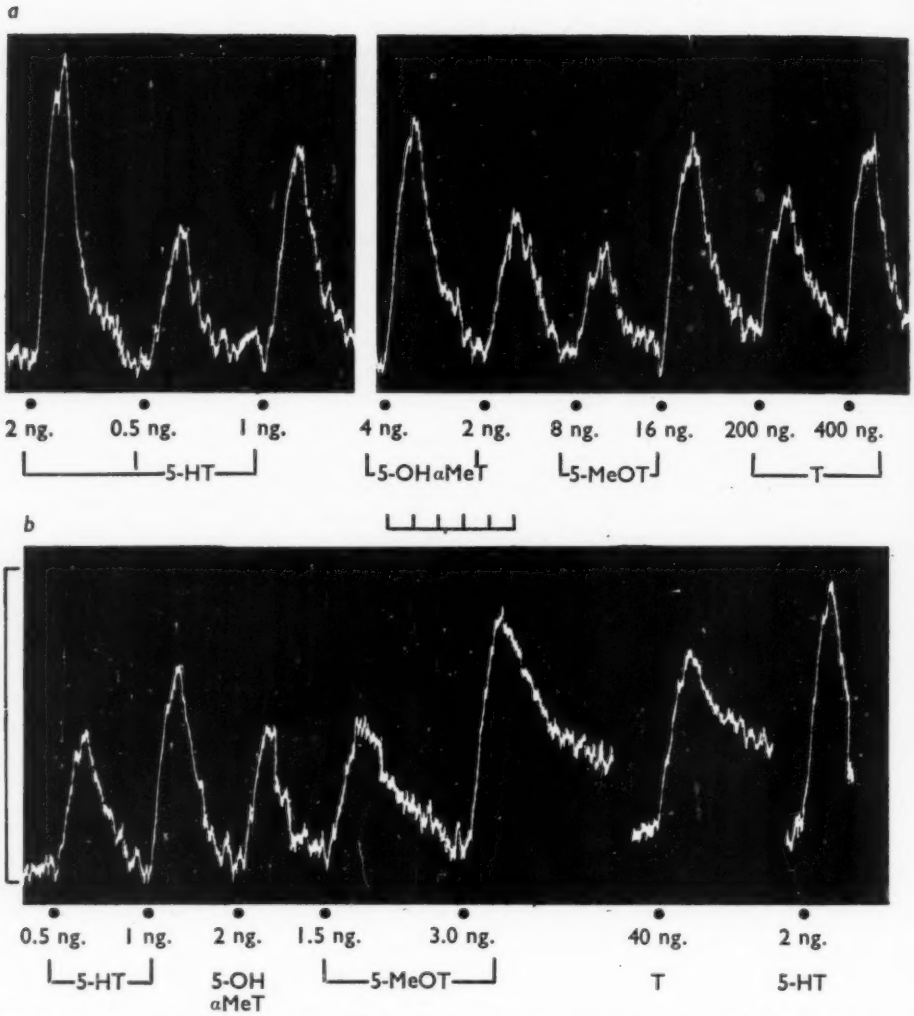


FIG. 2.—(*a*) Responses of the isolated stomach strip preparation to 5-hydroxytryptamine (***5-HT***), 5-hydroxy-α-methyltryptamine (***5-OHαMeT***, No. 24), 5-methoxytryptamine (***5-MeOT***, No. 19) and tryptamine (***T***, No. 1). (*b*) 60 min. after addition of phenyl*iso*propylhydrazine (0·1 μg./ml.) to the Tyrode solution. The contractions produced by ***5-HT*** and ***5-OHαMeT*** are unchanged. The effects of ***5-MeOT*** and ***T*** are potentiated in magnitude and duration. Time, min. Vertical scale, 10 cm.

dissociation constants and the oil/water partition coefficients were determined for representative members of the series (Table III). The *p*K values showed that the terminal amino group was almost completely ionized at physiological *p*H, but the ring hydroxyl group was in the unionized form. That the ring hydroxyl group contributed a great deal of oil insolubility to the molecule was shown by a comparison of the oil/water partition coefficients for tryptamine and 5-hydroxytryptamine at a low concentration. Tryptamine was almost as soluble in the oil as in the aqueous phase, but 5-hydroxytryptamine had a coefficient of 0.055, meaning that only 55 parts enter the oil phase out of every 1,000 in the aqueous phase.

Duration of Action

The rate of relaxation of the stomach strip after washing out the active drug probably depends upon the rate of detachment of the drug from the receptor site. This may be influenced by at least three factors, the affinity of the drug for the receptor, the rate of diffusion of the uncombined drug away from the receptor and destruction of the drug by an enzyme.

Figs. 1 and 2 show that after enzyme inhibition the contraction produced by tryptamine took much longer to pass off. Fig. 3 also illustrates that tryptamines which cannot be attacked by amine oxidase had a much longer duration of action than structurally similar compounds oxidizable by the

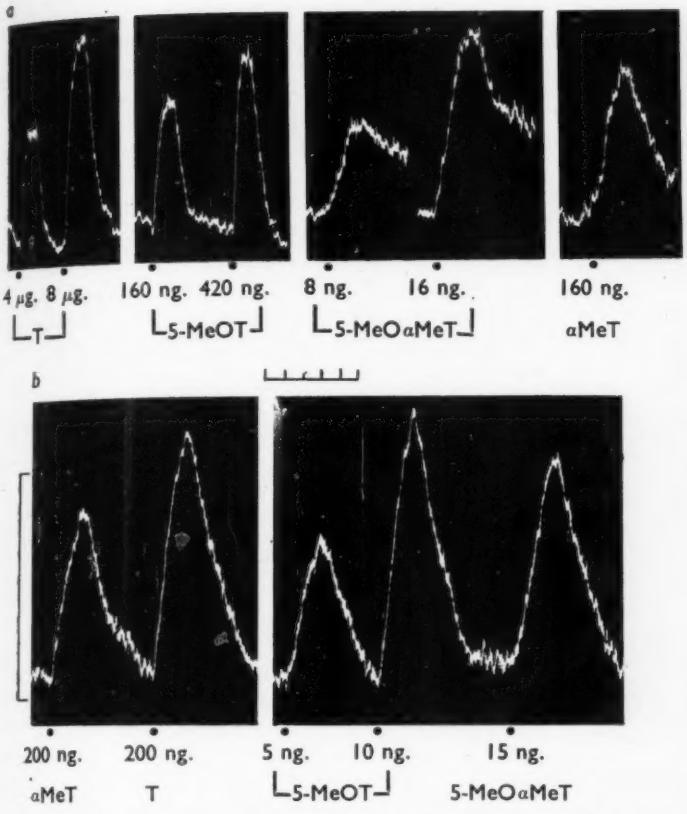


FIG. 3.—(*a*) Contractions of the isolated rat stomach strip preparation produced by tryptamine (***T***, No. 1), 5-methoxytryptamine (***5-MeOT***, No. 19), 5-methoxy-α-methyltryptamine (***5-MeOαMeT***, No. 20) and α-methyltryptamine (***αMeT***, No. 4). Note that the last two drugs produce effects which are much longer in duration. (*b*) A similar comparison, on a different preparation. Phenyl*iso*propylhydrazine had been added to the Tyrode solution 75 min. beforehand. The effects are comparable in duration, and tryptamine and 5-methoxytryptamine are now slightly more active than their α-methyl analogues. Time, min. Vertical scale, 10 cm.

enzyme. On the other hand, the relaxation of the tissue after 5-hydroxytryptamine was fairly rapid, relaxation after 5-hydroxy-α-methyltryptamine was also rapid and amine oxidase inhibitors did not affect the rate of relaxation after either substance (Fig. 2).

Blood Pressure

Intravenous injections of 5-hydroxytryptamine, 5-methoxytryptamine and tryptamine were compared for their pressor effects in the spinal cat, using infrequent injections to avoid tachyphylaxis. After the injection of either iproniazid or phenyl*iso*propylhydrazine, the effect of 5-hydroxytryptamine did not change substantially. The effects of both tryptamine and 5-methoxytryptamine were potentiated both in magnitude and in duration of action (Fig. 4).

Discussion

In the Warburg flask, both 5-hydroxytryptamine and tryptamine are good substrates for liver amine oxidase. It has now been shown that the rat fundus, when finely ground up, contained an enzyme which behaved like amine oxidase and oxidized both 5-hydroxytryptamine and tryptamine. It also broke down 5-methyltryptamine. Other tryptamines with substituents in the 5-position of the indole ring should also be substrates for amine oxidase, but it was not possible to study these because of the limited quantity of the compounds available.

If the enzyme present in the homogenate is also active in the whole tissue, then inhibition of the enzyme should lead to a potentiation of the action of substances normally oxidized by it. Such a potentiation is well established for the action of acetylcholine on smooth muscle after inhibition of cholinesterase. It has also been suggested that the potentiation of the action of adrenaline-like compounds by ephedrine and cocaine may be due to amine oxidase inhibition (see Blaschko, 1952, 1954), but this hypothesis has to be reconsidered in the light of the recent work of Burn and Rand (1958), who postulate that substances such as ephedrine bring about their sympathomimetic effects through the release of noradrenaline.

Brown and Gillespie (1957) have suggested that the tissue receptors for noradrenaline were also responsible for its destruction. That this concept cannot hold for tryptamine receptors is shown by the fact that blocking amine oxidase activity substantially increased the action of tryptamine in the rat fundus. Indeed, this potentiation after enzyme inhibition is the first clear evidence that amine oxidase limits the amount of the active substance reaching the receptor site in the whole tissue. This potentiation, sometimes by more than twentyfold and comparable in magnitude to acetylcholine potentiation after cholinesterase inhibition, provided an opportunity to study the interrelation between the activity of a drug on the rat fundus and the role of amine oxidase in modifying this activity. A comparison of the relative activities of tryptamine (No. 1) and

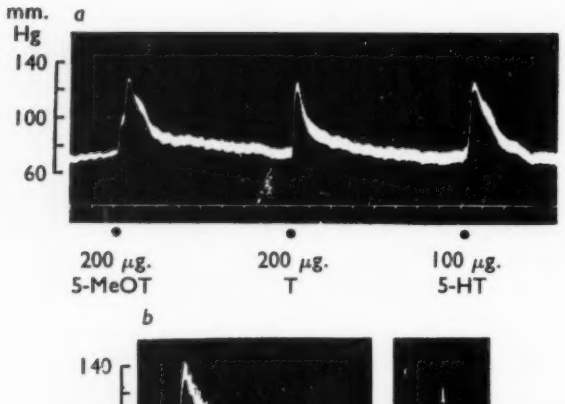


FIG. 4.—Blood pressure recording from a spinal cat (3·3 kg.). (*a*) The effects of 5-methoxytryptamine (***5-MeOT***, No. 19), tryptamine (***T***, No. 1) and 5-hydroxytryptamine (***5-HT***). (*b*) After phenyl*iso*propylhydrazine (1 mg./kg.). Note the potentiation and prolongation of the tryptamine effect. A second dose of phenyl*iso*propylhydrazine (mg./kg.) was given before the 5-hydroxytryptamine injection. (*c*) After a third dose of phenyl*iso*propylhydrazine (1 mg./kg.). Note the prolongation of the effect of ***5-MeOT***. Time, min.

α-methyltryptamine (No. 4) before and after enzyme inhibition afforded a good example. It is known that an α-methyl group in the side-chain prevents oxidation by amine oxidase. The fact that in the normal fundus α-methyltryptamine was 10 times more active than tryptamine can be explained by the action of amine oxidase in limiting the concentration of tryptamine around the receptor site. The true potency of these two substances was only seen, therefore, after amine oxidase inhibition, when tryptamine was found to be slightly more active than α-methyltryptamine. This means that at the muscle receptor tryptamine is more potent than α-methyltryptamine, but that without amine oxidase inhibition the concentration of tryptamine at the receptor is kept at a low level by destruction. In fact in the absence of amine oxidase activity, tryptamine was the most potent of all the tryptamines studied in which there were no substituents on the indole ring (Table II). None of the other compounds "fits" the receptor as well as does tryptamine, but they appear more potent when amine oxidase is active because they are oxidized to a lesser degree. An estimate of the ability of amine oxidase to inactivate these compounds in the isolated organ is shown in Table II by the degree of potentiation after enzyme inhibitors. It is interesting to note that the substitution of the hydrogen atoms of the terminal amino group by alkyl groups is as effective in preventing oxidation by the enzyme as is the introduction of a methyl group on the α-carbon atom. The *N'N'*-dimethyl and *N'N'*-diethyl derivatives (Nos. 10 and 11) are, however, less active at the muscle receptor: it is not until the terminal amino group is loaded with two propyl groups (No. 12) that the potency returns almost to match tryptamine itself.

Having found that tryptamine was limited in its action by the amine oxidase present in the tissue, and that this could be overcome either by the introduction of a methyl group on the α-carbon atom or of two propyl groups on the terminal amino group without much loss in inherent activity, it was expected that the same would hold true for 5-hydroxytryptamine. It was therefore puzzling to find that 5-hydroxy-α-methyltryptamine (No. 24) and 5-hydroxy-*N'N'*-dipropyltryptamine (No. 29) were less active than 5-hydroxytryptamine. An explanation is found in the observation that inhibition of amine oxidase in the fundus strip does not appreciably potentiate the action of 5-hydroxytryptamine. Because of the gradual increase in general sensitivity of the rat fundus to all drugs as the experiment proceeds, a slight potentiation of the action of 5-hydroxytryptamine could easily be missed. Such a potentiation would show itself, however, as a value of less than 1.0 in Table II. 5-Hydroxy-α-methyltryptamine (No. 24), for example, appears less active in comparison with 5-hydroxytryptamine after amine oxidase inhibition (potentiation factor = 0.7). Using 5-hydroxy-α-methyltryptamine as the reference standard, 5-hydroxytryptamine could be said to be increased in potency after amine oxidase inhibition by a factor of 1.4. This is the maximum possible potentiation from the values in Table II, and will be regarded as insignificant for the purpose of this discussion.

In contrast to the lack of potentiation of 5-hydroxytryptamine on the fundus strip after amine oxidase inhibition, the finely ground-up preparation oxidized 5-hydroxytryptamine at about the same rate as tryptamine. The only conclusion that can be drawn is that in the whole preparation the tissue receptors and the amine oxidase are spatially arranged so that tryptamine reaches both but 5-hydroxytryptamine only reaches the receptors. This would account for the inability of structural modification and amine oxidase inhibition to increase the potency of 5-hydroxytryptamine.

There are at least two ways in which orientation of the receptors and the enzyme might account for the results. First, it could be postulated that there are different receptors for tryptamine and for 5-hydroxytryptamine. The tryptamine receptors would have to be devoid of enzyme in the vicinity. In considering this hypothesis, it must be remembered that substances such as 5-methoxytryptamine (No. 19), 5-chlorotryptamine (No. 21) and 5-methyltryptamine (No. 22) are all potentiated in their action in the presence of an amine oxidase inhibitor, or by the introduction of a methyl group on the α-carbon atom. It would indeed be strange if all of these 5-substituted compounds combined preferentially with "tryptamine receptors" and not with "5-hydroxytryptamine receptors."

The second hypothesis is much more attractive and involves only a diffusion barrier which excludes 5-hydroxytryptamine but allows tryptamine to penetrate. The simplest explanation is that the cell surface is the diffusion barrier and that the amine oxidase is contained inside the cell.

There is abundant evidence that, in general, amine oxidase is an intracellular enzyme associated with cytoplasmic granules (Blaschko, 1952) and there is no reason to suppose that the rat fundus differs from other tissues in this respect. The *p*K values for 5-hydroxytryptamine, which agree with those published by Rapport, Green, and Page (1948), show that the ring hydroxyl group is only ionized to the extent of 0.005% at physiological *p*H. Nevertheless, the polar properties of the unionized ring hydroxyl group substantially alter the physical characteristics of tryptamine-like substances (compare benzene and phenol), as is shown by the low oil/water partition coefficient of 5-hydroxytryptamine as compared with tryptamine. Meyer (1899) and Overton (1901) first used the olive oil/water partition coefficient as a guide to the penetration of substances into the cell. It is now generally accepted that drugs with high fat solubility enter the cell easily, and that those with low fat solubility do not unless a special transport mechanism exists (see Brodie and Hogben, 1957).

It would be expected then, simply on the basis of fat solubility, that tryptamine would enter the cell far more easily than 5-hydroxytryptamine, unless an active transport mechanism exists for 5-hydroxytryptamine. Born (1958) has shown that platelets, which store 5-hydroxytryptamine in cytoplasmic granules, must have an active transport mechanism, but this seems to be a specialized case. All other evidence points to the fact that 5-hydroxytryptamine has low lipid solubility and does not penetrate cells. For instance, when administered parenterally, 5-hydroxytryptamine does not cross the blood-brain barrier unless huge doses are used (Brodie and Hogben, 1957). In contrast, central disturbances similar to those caused by mescaline and lysergic acid diethylamide have been reported after doses of 1 mg./kg. of *N'N'*-dimethyltryptamine and *N'N'*-diethyltryptamine (Szára, 1957), so these substances must cross the blood-brain barrier.

If it is accepted that tryptamine enters cells and 5-hydroxytryptamine does not, the results presented in this paper are explicable. The contraction of the rat fundus is brought about by a surface action of tryptamine-like compounds, an action not dependent upon cell penetration. At the same time, those tryptamine derivatives which can enter the cell and which are inactivated by the intracellular amine oxidase are normally limited in their action because the surface concentration is constantly being reduced by intracellular inactivation. Substances with a polar hydroxyl group attached to the indole ring, such as 4-, 5- and 6-hydroxytryptamine, do not enter the cell and are therefore immune from the action of amine oxidase. Inhibition of amine oxidase will not potentiate their action on the rat fundus. If, however, the fundus is homogenized and the integrity of the cell destroyed, then the amine oxidase can act on 5-hydroxytryptamine. In substances in which the polar nature of the hydroxyl group is removed, such as 5-methoxytryptamine, cell permeability is restored and the action of such compounds on the rat fundus is once more limited by the intracellular enzyme.

The differences in the persistence of the tryptamine analogues after washing out the bath are also interesting. First, only those which can penetrate the cell have a prolonged action after the drug has been removed from the bath. The relaxation rate of the fundus is similar after either 5-hydroxytryptamine or 5-hydroxy-α-methyltrypt-

amine (No. 24). Secondly, of those drugs which penetrate the cell, only those which are unaffected by amine oxidase activity produce a persistent contraction (Figs. 1, 2, and 3). Thirdly, if the amine oxidase within the cell is inhibited, then all the compounds which diffuse inside have a similar persistent action. It would seem, therefore, that in order for a drug to produce a contraction of the rat fundus which persists after removal of the drug from the bath, it must diffuse into the cell and be protected from the action of the intracellular enzyme responsible for inactivation.

This theory of selective membrane permeability as an explanation of the results presented in this paper seems to be the most reasonable. However, the cell membrane itself is not the only possible diffusion barrier, for the cytoplasmic particles may also present a diffusion barrier to polar molecules. Indeed, it would be difficult to explain how a population of cytoplasmic granules can exhibit both amine oxidase activity and a capacity to store 5-hydroxytryptamine if some intracellular diffusion barrier was not present.

The rôle of amine oxidase in the body has been enigmatic for many years. It was first thought to be the mechanism for inactivation of adrenaline and, later, noradrenaline. However, the inability to potentiate the actions of adrenaline and noradrenaline in the whole animal by amine oxidase inhibitors (Corne and Graham, 1957) has made this rôle doubtful. When 5-hydroxytryptamine was identified both as a naturally occurring substance and as a substrate for amine oxidase, it was generally thought that a new and more likely rôle had been discovered. Even so, the actions of 5-hydroxytryptamine in the whole animal are not potentiated by inhibition of the enzyme, although the stores of 5-hydroxytryptamine in bound form increase (Udenfriend, 1958). The significance of amine oxidase as an intracellular enzyme and of the possible natural substrates being excluded from the cell has, in general, been ignored. Presented in this context, the function of amine oxidase would seem to be directed towards preventing unnatural but active compounds from having an action on, and being stored within, the cell. This concept of amine oxidase as a protective enzyme, to reduce the concentration of unwanted but active compounds around the extracellular receptors has been discussed by Blaschko (1952). It becomes much stronger if the amine oxidase is unable to inactivate the naturally occurring substrate, 5-hydroxytryptamine, because of a diffusion barrier. This is certainly true for the isolated rat fundus. The degree and duration of action of both tryptamine (No. 1) and 5-methoxytryptamine (No. 19) are also potentiated in the spinal cat by amine oxidase inhibition, whereas the action of 5-hydroxytryptamine is not. If it is generally true for all tissues, then a relatively low conversion of injected or endogenous 5-hydroxytryptamine into urinary 5-hydroxyindoleacetic acid would be expected. Keglević-Brovet, Supek, Kveder, Iskrić, and Kečkeš (1958) have shown that only about 20% of injected labelled 5-hydroxytryptamine is excreted in the form of 5-hydroxyindoleacetic acid, a figure similar to that arrived at by Erspamer (1956). Further work on this point is needed. In the whole animal, for instance, it would be expected that for any given effect of 5-hydroxytryptamine which is limited by the action of amine oxidase, the effect of 5-hydroxy-α-methyltryptamine (No. 24) or 5-hydroxy-*N'N'*-dipropyltryptamine (No. 29) would be much greater.

Even though amine oxidase may be barred from oxidizing 5-hydroxytryptamine directly, it is interesting to speculate on the other possible inactivation processes. It has recently been shown that one of the first steps in the inactivation of adrenaline and noradrenaline is the methylation of one of the ring hydroxyl groups (Axelrod, Senoh, and Witkop, 1958 ; Axelrod and Tomchick, 1958). It is tempting to postulate that methoxylation enables the previously highly polar amine to penetrate the cell and there be oxidized by the intracellular amine oxidase. This sequence would explain why amine oxidase inhibitors do not potentiate the actions of adrenaline and noradrenaline in the body, for the methoxylated amine has much less activity than the parent compound (Gillespie, Evarts, Fleming, and Sjoerdsma, 1958).

A similar sequence is less likely for 5-hydroxytryptamine, because 5-methoxytryptamine is highly active. If 5-hydroxytryptamine were methoxylated and then inactivated by amine oxidase, inhibition of amine oxidase would lead to an accumulation of 5-methoxytryptamine and an apparent potentiation of the effects of 5-hydroxytryptamine. As this does not occur, it is unlikely that methoxylation plays an important rôle in 5-hydroxytryptamine metabolism. If an intermediate product is formed, it must be both more lipid soluble and substantially less active than 5-hydroxytryptamine.

The ability to modify the tryptamine or 5-hydroxytryptamine molecule in such a way as to preserve its activity but to make it resistant to attack by amine oxidase may have important ramifications. The central activity of tryptamine-like drugs has received much attention in the last

few years, but work has been hampered and hard to interpret because 5-hydroxytryptamine does not cross the blood-brain barrier. The use of an analogue of tryptamine, able to penetrate cells but resistant to amine oxidase activity, might give interesting results. 5-Methoxy-α-methyltryptamine (No. 20), *N'N'*-dipropyltryptamine (No. 12), and α-methyltryptamine (No. 4) are the most potent of these compounds. Similarly, if 5-hydroxytryptamine does have peripheral actions which are limited by amine oxidase activity, the α-methyl or *N'N'*-dipropyl derivatives of 5-hydroxytryptamine would be useful tools.

There are several other points of interest which emerge from a comparison of the activities and structures of these compounds. Methylation of the ring hydroxyl group, for instance, only reduced the potency by a factor of about two (after amine oxidase inhibition). Thus, 5-methoxytryptamine (No. 19) had an equipotent molar ratio of 1.7, compared to 5-hydroxytryptamine=1. 6-Hydroxytryptamine (No. 16) had an equipotent molar ratio of 560 and this was reduced to 950 for 6-methoxytryptamine (No. 17), again a ratio between the hydroxy and methoxy derivatives of 1.7. 5:6-Dimethoxytryptamine (No. 18) had an equipotent molar ratio of 147. Assuming that the hydroxy derivative would have a potency 1.7 times as great, then the equiactive molar ratio of 5:6-dihydroxytryptamine can be predicted to be 87. This does not support the speculation that 5:6-dihydroxytryptamine would be more potent than 5-hydroxytryptamine (Dalgliesh, 1958). Should a highly active compound containing two ring hydroxyl groups be made, 4:5-dihydroxytryptamine would be the more interesting, for 4-hydroxytryptamine (No. 15) had one half the activity of 5-hydroxytryptamine, whereas 6-hydroxytryptamine (No. 16) had only one-sixhundreth of the activity.

The importance of the substituent in the 5-position of the indole ring is shown by the fact that tryptamine has less activity than 5-hydroxy-, 5-methoxy-, 5-chloro-, or 5-methyl-tryptamine (Nos. 14, 19, 21, and 22). That 4-hydroxytryptamine should be of comparable activity to 5-hydroxytryptamine, whereas 6-hydroxytryptamine was so much weaker emphasizes again that the 4- and 5-position of the indole ring may be important points of attachment for tryptamine derivatives to the receptor site. At the other end of the molecule, the importance of the length of the side-chain is illustrated by comparison of the activities of indolemethylamine, tryptamine and homotryptamine (Nos. 2, 1, and 3). As would be expected, the order of potency is tryptamine, homotryptamine and, very much weaker, indolemethylamine. If the terminal amino group is a second point of attachment of the molecule to the receptor site, the rotation of the longer side-chain of homotryptamine will include this second point of attachment, whereas the side-chain of indolemethylamine will be too short.

Further information on the possible points of attachment of tryptamine derivatives to the receptor site can be adduced from a comparison of the activities of *N'N'*-dimethyltryptamine (No. 10) and 2:*N'N'*-trimethyltryptamine (No. 13). The introduction of the methyl group at the 2-position of the indole ring brings about a tenfold reduction in activity. It will also limit free rotation of the side-chain by steric hindrance. If the reduction in activity is assumed to be due to steric hindrance, then it can be deduced that the point of attachment of the terminal amino group is best fitted when the side-chain is rotated to a position fairly close to the indole ring, at a maximum distance away from the ring hydroxyl group. These two probable points of attachment of 5-hydroxytryptamine to the receptor site may not, however, be the only ones. The importance of the ring nitrogen, for instance, can only be assessed when simpler compounds which do not contain it are available.

Finally, by the use of amine oxidase inhibitors, it is now possible to distinguish biologically between 5-hydroxytryptamine and other tryptamine derivatives which may be present in tissue extracts.

This work was made possible by the gifts of many compounds otherwise unobtainable. I would particularly like to thank Dr. R. B. Barlow for compounds Nos. 7, 8, 9, 10, 11, 12, 13, 27, 28, and 29, all of which were used as the hydrochlorides except Nos. 10 (fumarate) and 27 (creatinine sulphate); Dr. G. Curzon for Nos. 19 (hydrochloride), 27 (base), 30 and 31; Dr. J. B. Jepson for Nos. 7, 8, 11, 17, and 18 as hydrochlorides, Nos. 2, 3, and 26 as hydrogen oxalates, Nos. 15 and 16 as creatinine sulphates, No. 6 as acetate; and Dr. A. Spinks of I.C.I. (Pharmaceutical Division) for Nos. 4, 5, 20, 21, 22, 23, 24, and 25 as hydrochlorides; Nos. 24 and 25 were also supplied as dipicrates. The synthesis of these I.C.I. compounds has been described by Young (1958).

Tryptamine hydrochloride and 5-hydroxytryptamine creatinine sulphate were purchased from the British Drug Houses and May and Baker respectively. Iproniazid was a gift from Roche Products Ltd.

I am also indebted to Professor W. D. M. Paton and Dr. H. Blaschko for their interest and for many helpful discussions.

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ACTIONS OF SOME ANALOGUES OF TRYPTAMINE ON THE ISOLATED RAT UTERUS AND ON THE ISOLATED RAT FUNDUS STRIP PREPARATIONS

BY

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Some 3-(2-dialkylaminoethyl)-, 3-(2-alkylaminoethyl)-, and 3-(2-dialkylaminoethyl)-2-methylindoles have been synthesized and tested, along with 5-benzyloxygramine, 5-benzyloxy-3-(2-dimethylaminoethyl)-, 3-(2-aminopropyl)- and 3-(2-aminobutyl)-indoles, on the rat uterus and rat fundus strip. 5-Benzyloxy-3-(2-dimethylaminoethyl)indole, even though it contains an ethylene side-chain, was a less potent antagonist of 5-hydroxytryptamine than was 5-benzyloxygramine. The remaining compounds were still less active. There are differences between the ability of some of them to antagonize 5-hydroxytryptamine and to antagonize tryptamine. 3-(2-Dimethylaminoethyl)-2-methylindole, in particular, shows a considerable degree of specific antagonism of 5-hydroxytryptamine on both tissues.

Nearly all the compounds stimulate the preparations, some combining antagonism of 5-hydroxytryptamine at low concentrations with stimulant activity at higher concentrations. The most active compound is 3-(2-dipropylaminoethyl)indole. On the rat fundus strip this has, on a molar basis, 1/40th of the stimulant activity of 5-hydroxytryptamine, and is 20 times as active as tryptamine: on the rat uterus it is only 1/200th as active as 5-hydroxytryptamine, and equal in activity to tryptamine.

From quantitative studies of antagonists of 5-hydroxytryptamine on the rat uterus, Gaddum, Hameed, Hathway, and Stephens (1955) concluded that, in compounds related to 3-aminomethylindole and 3-(2-aminoethyl)indole (tryptamine), antagonist activity was increased by: (1) Introduction of two methyl groups on the amino group in the side-chain. (2) Introduction of a methyl group in the 2-position of the indole nucleus (this effect was also noted by Woolley and Shaw, 1953, in another series of substituted indoles). (3) Introduction of a benzyloxy group in the 5-position of the indole nucleus of gramine [they did not study 5-benzyloxy-3-(2-dimethylaminoethyl)indole].

In a search for other antagonists of 5-hydroxytryptamine, we have prepared and tested a number of analogues of tryptamine, some of which contain these structural features (Table I). There are three types of compound: (1) 3-(2-Dialkylaminoethyl)indoles, (2) 3-(2-alkylaminoethyl)indoles and (3) 3-(2-dialkylaminoethyl)-2-methylindoles. We have included in our work 3-(2-aminopropyl)indole (α-methyltryptamine), 3-(2-aminobutyl)indole (α-ethyltryptamine) and 5-benzyloxygramine (Gaddum, Hameed, Hathway, and Stephens, 1955) and also 5-benzyloxy-3-(2-dimethylaminoethyl)indole (Speeter and Anthony, 1954), whose pharmacological properties do not appear to have been extensively investigated.

The substances have been tested for both stimulant and antagonistic properties on the rat uterus and on the rat fundus strip (Vane, 1957).

METHODS

Drugs.—The compounds were all prepared by the method of Speeter and Anthony (1954). Indole, 2-methylindole or 5-benzyloxyindole, dissolved in ether and treated with oxalyl chloride, gave the 3-glyoxalyl chloride which was converted to the appropriate amide. This was reduced with lithium aluminium hydride in ether or, more usually (as it is a better solvent for the amide), in tetrahydrofuran. Excess lithium aluminium hydride was destroyed with water, the solvent decanted off and the residue washed with ether. The combined extracts were dried and concentrated and the base was distilled at 10^{-3} to 10^{-4} mm. of Hg pressure. The clear oil or

solid distillate was converted into the hydrochloride or, if this would not crystallize, into the fumarate.

The 5-benzyloxygramine was the sample used by Gaddum, Hameed, Hathway, and Stephens (1955). Although the drugs are referred to as if bases, a suitable salt (Table I) was invariably used.

Preparations.—The isolated uterus was set up in de Jalon solution in a 2 ml. bath, exactly as used by Gaddum and Hameed (1954). The temperature was 30°. It was found that portions of uterus could be stored at 4° for up to two days without loss of sensitivity to either 5-hydroxytryptamine or tryptamine.

The isolated rat fundus strip preparation was cut exactly as described by Vane (1957) but mounted, as subsequently recommended by Vane (personal communication), with a pendular auxotonic lever (Paton, 1957). In some experiments, the magnification of the lever was 16 (as originally described by Vane) and the load on the muscle was 1 g. In other experiments, where it was desired to record a maximum contraction, the magnification was only 4 and only three-quarters of the full length of the dissected fundus strip was used. In these experiments the load on the muscle was 0.5 g. The preparation was suspended in Tyrode solution containing a concentration of 10^{-7} hyoscine hydrobromide which prevented interference from acetylcholine-like effects and also (Vane, personal communication) produced a much steadier base-line. Pure oxygen was bubbled through the bath, which had a volume of 5 ml. The temperature was 37°.

Experimental Procedures

Qualitative

Rat Uterus.—All the drugs were tested on the rat uterus in concentrations varying from below the smallest which produced an effect (usually 10^{-7} to 10^{-6} M) up to 10^{-4} M. The drugs were added directly to the bath in a volume not exceeding 0.2 ml. and allowed to act for 45 sec. The preparation was then washed and allowed 3.25 min. for recovery; the interval between doses was thus 4 min. Control contractions of about 50% of the maximum possible response were obtained with 5-hydroxytryptamine, tryptamine (in some experiments) and acetylcholine. These drugs were added in that order, first in the absence of the test drug, then in its presence (it was maintained in the bath by adding it freshly after each wash), then again in its absence.

After high doses of 5-hydroxytryptamine, the preparation is much less sensitive to smaller ones. To see whether such an antagonism of 5-hydroxytryptamine was shown by these other compounds, the responses to control doses of 5-hydroxytryptamine and of acetylcholine were observed before and after a high concentration (10^{-4} M) of the test drug had been left in the bath for 5 to 10 min.

Rat Fundus Strip.—The drugs were added directly to the bath in a volume not exceeding 0.2 ml. and allowed to act for 90 sec. The preparation was

TABLE I

COMPOUNDS AND STIMULANT ACTIVITY

Microanalyses are by Dr. J. W. Minnis (Department of Biochemistry); melting points were taken on a Kofler hot stage. An asterisk indicates that Speeter and Anthony (1954) record m.p. 154–5°. EtOH = ethanol; MEK = ethyl methyl ketone; Et_2O = ether. † Indicates that the dose/response curve was not parallel to that of 5-hydroxytryptamine and at high doses a value as extreme as 20,000 : 1 was obtained. M indicates that estimates were determined by matching (see text).

Comp. No.	Ring Sub-stituent	Side-chain (R) (Trivial Name)	Salt	Crystallized from	m.p. (°C.)	Found			Theory			Stimulant Activity Equipotent Molar Ratios: Mean ±s.e.			
						C	H	N	C	H	N	Rat Uterus 30°	No. of Expts.	Rat Fundus Strip 37°	No. of Expts.
1	—	$-CH_2-CH_2-N(CH_3)_2$ (*N*′*N*′-Dimethyltryptamine)	Fumarate Picrate	EtOH/MEK/Et_2O EtOH	152–152·5 169·5–170·5	63·3 51·6	6·81 4·51	9·40	63·2 51·8	6·65 4·60	9·21	†1,000 (M)	2	350±100	4
2	—	$-CH_2-CH_2-N(C_2H_5)_2$ (*N*′*N*′-Diethyltryptamine)	HCl Picrate	EtOH/MEK/Et_2O EtOH	172–3 170·5	66·4 54·2	8·37 5·03	10·7	66·5 53·9	8·40 5·22	11·1	500 (M)	2	112±14	3
3	—	$-CH_2-CH_2-N(C_3H_7)_2$ (*N*′*N*′-Dipropyltryptamine)	HCl	EtOH/MEK/Et_2O	176–8	68·7	8·85		68·5	8·64		200±23	3	40±9	3
4	—	$-CH_2-CH_2-N(isoC_3H_7)_2$ (*N*′*N*′-Diisopropyltryptamine)	HCl	EtOH/MEK	195·5–196·5	68·6	8·74		68·5	8·64		520±104	3	53±8	4
5	—	$-CH_2-CH_2-N(C_4H_9)_2$ (*N*′*N*′-Dibutyltryptamine)	HCl	EtOH	184–5	70·1	9·05		70·0	9·17		500 (M)	2	244±65	4
6	—		HCl Picrate	EtOH/MEK/Et_2O EtOH	225–7 174–174·5	62·0 55·2	7·50 4·88	10·3	55·1 53·1	7·64 5·08	10·8	1,000 (M)	1	1,000 (M)	1
7	—														

4	—	$-CH_2-CH_2-N(isoC_3H_7)_2$ (N',N'-Diisopropyltryptamine)	HCl	EtOH/MEK	195·5–196·5	68·6	8·74		68·5	8·64		520±104	3	53±8	4
5	—	$-CH_2-CH_2-N(C_4H_9)_2$ (N',N'-Dibutyltryptamine)	HCl	EtOH	184–5	70·1	9·05		70·0	9·17		500 (M)	2	244±65	4
6	—		HCl	EtOH/MEK/Et_2O	173–174·5	67·9	8·88	10·3	68·1	7·64	10·6				
7	—		Picrate	EtOH	174–174·5	55·2	4·88		55·1	5·08					
8	—	$-CH_2-CH_2-N$	HCl	EtOH/MEK	214–5	63·2	7·02		63·1	7·20		> 10,000 (M)	2	> 75,000	2
			Picrate	EtOH	202–3	52·4	4·43		52·3	4·62					
9	—	$-CH_2-CH_2-NH(CH_3)$ (N'-Methyltryptamine)	HCl	EtOH/MEK/Et_2O	181·5–182·5	62·8	6·91	13·3	62·7	7·20	13·3	2,000 (M)	2	2,400±98	3
			Picrate	EtOH	193–4	51·2	4·45		50·6	4·26					
10	—	$-CH_2-CH_2-NH(C_2H_5)$ (N'-Ethyltryptamine)	HCl	EtOH/MEK/Et_2O	183–4	64·2	7·56	12·7	64·1	7·65	12·5	2,000 (M)	2	350±29	3
			Picrate	EtOH	187–9	51·9	4·40		51·8	4·60					
11	—	$-CH_2-CH_2-NH(C_3H_7)$ (N'-Propyltryptamine)	HCl	EtOH/MEK/Et_2O	184–185·5	65·5	8·07		65·4	8·05		2,000 (M)	2	490±85	4
12	2-CH_3	$-CH_2-CH_2-N(CH_3)_2$ (2: N',N'-Trimethyltryptamine)	HCl	EtOH/MEK	211–2	65·2	7·60		65·4	8·05		> 20,000 (M)	2	6,665±1,330	3
			Picrate	EtOH	176·5	52·8	4·93		52·9	4·92					
13	2-CH_3	$-CH_2-CH_2-N(C_2H_5)_2$ (N',N'-Diethyl-2-methyltryptamine)	HCl	EtOH/MEK/Et_2O	217–8	67·8	8·87		67·5	8·72		1,000 (M)	2	280±42	4
			Picrate	EtOH	174–5	55·0	5·30		54·9	5·50					
14	2-CH_3	$-CH_2-CH_2-N(C_3H_7)_2$ (2-Methyl-N',N'-dipropyltryptamine)	HCl	EtOH/MEK	227–8	69·4	9·27		69·3	9·26		630 (M)	2	70±10	3
15	5-OC_2H_5	$-CH_2-CH_2-N(CH_3)_2$ (5-Benzyloxy-N',N'-dimethyltryptamine)	HCl	EtOH/MEK/Et_2O	159–161*	69·3	6·73	8·42	68·9	7·03	8·47	> 20,000	2	625±135	4
16	5-OC_2H_5	$-CH_2-N(CH_3)_2$ (5-Benzyloxygramine)												25,000	1
17	—	$-CH_2-CH(CH_3)-NH_2$ (α-Methyltryptamine)	HCl									800		50±14	3
18	—	$-CH_2-CH(C_2H_5)-NH_2$ (α-Ethyltryptamine)	HCl									> 20,000	2	10,000	2
19	—	$-CH_2-CH_2-NH_2$ (Tryptamine)	HCl									210±30	4	933±33	3
20	5-OH	$-CH_2-CH_2-NH_2$ (5-Hydroxytryptamine)										1		1	

washed and stretched for 60 sec. and allowed a further 2.5 min. for recovery; the interval between doses was thus 5 min. On this preparation, the effects of low concentrations of the drugs were of particular interest. A concentration, too small to produce a contraction (though often disturbing the base line), was maintained in the bath for from 15 to 45 min. to see how this affected the responses to repeated doses of 5-hydroxytryptamine and tryptamine.

The highest concentrations tested on this preparation were not usually greater than those which produced a maximum contraction (around 10^{-5} M) as, on the occasions when they were tested, concentrations above this did not give rise to repeated contractions or to non-specific inhibition, such as was observed on the rat uterus.

Quantitative

Antagonistic Activity. — This was determined by measuring the dose ratio and, if possible, the drug ratio of the drug to 5-hydroxytryptamine exactly as described by Gaddum, Hameed, Hathway, and Stephens (1955). As in their experiments, the antagonists were maintained in the bath for 1 hr. The procedure with the rat fundus strip only differed from that with the rat uterus in that the interval between doses was 5 min. instead of 4 min. (see above). In all experiments on antagonistic activity with the rat fundus strip, control responses were obtained with acetylcholine (in large doses because of the hyoscine present).

Stimulant Activity.—Dose/response curves for all the compounds were obtained on the rat fundus strip and for some of the compounds on the rat uterus. On any one preparation only two curves were obtained, one for 5-hydroxytryptamine and one for the test drug. In these experiments it was necessary with some of the drugs to modify the cycle of events. On the rat uterus it was a long time before the effects of certain compounds wore off and the controls returned to normal. On the rat fundus strip it was sometimes necessary to allow up to 3 min. for the contraction to reach its maximum and also to allow even up to 45 min. stretching to assist recovery. If insufficient time were allowed for recovery, the responses were reduced and the dose/response curves consequently appeared to flatten out. This

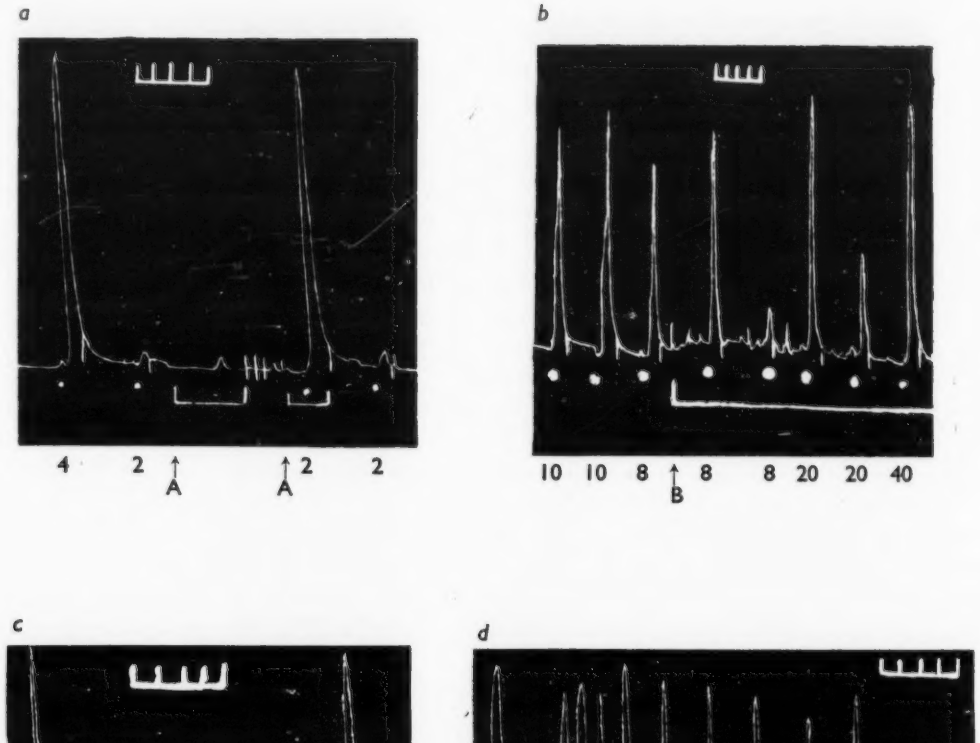

FIG. 1.—Isolated rat uterus preparation. Time, min. (*a*)—Synergism with 5-hydroxytryptamine (**5-HT**) added at white dots. At **A**, a concentration of $4{\cdot}8 \times 10^{-6}$ M 3-(2-dibutylaminoethyl)indole was maintained in the bath for the period indicated. By itself it had no effect, but when added with 2 ng. of **5-HT (4)** the response was about as big as that to 4 ng. of **5-HT (2)** previously. (*b*)—Antagonism of 5-hydroxytryptamine (**5-HT**) added at white dots. At **B** a concentration of 4×10^{-6} M 3-(2-diethylaminoethyl)indole was maintained in the bath. After 18 min. 40 ng. of **5-HT (40)** produced a response equivalent to 10 ng. of **5-HT (10)** previously. **8**=8 ng. of **5-HT**. (*c*)—Stimulant action. 3-(2-di*iso*propylaminoethyl)indole was added in a concentration $4{\cdot}5 \times 10^{-6}$ M at **C** and 9×10^{-6} M at C_1. Numerals indicate ng. of 5-hydroxytryptamine added. (*d*)—Repeated contractions. At **D** a concentration of 10^{-5} M 3-(2-dipropylaminoethyl)indole maintained in the bath for the period indicated. The repeated contractions persisted in spite of washing at **W**. **10**=10 ng. of 5-hydroxytryptamine. (*e*)—Profound depression. Responses to 500 ng. of carbachol (at white dots with **Cc** below them) and 5-hydroxytryptamine (**5-HT**) at white dots, the numerals below which indicate ng. of **5-HT**, are shown before and after treatment with 3-(2-dipropylaminoethyl)indole in a concentration at **E** of $3{\cdot}6 \times 10^{-5}$ M and E_1 of $7{\cdot}2 \times 10^{-5}$ M maintained for the period

gave the misleading impression that the compounds were partial antagonists (Stephenson, 1956). With the rat fundus strip it was necessary to reduce the magnification of the lever, as described above.

After every test contraction (whether of 5-hydroxytryptamine or of the drug), small control doses of 5-hydroxytryptamine were given and the experiment was continued only when the responses to these were normal. Because of the persistence of the effects of high doses, the doses were given in increasing order of magnitude rather than in any random manner.

The dose/response curves were examined by eye to see if they were parallel, and, as they were (with one dubious exception), it was possible to evaluate the equipotent molar ratio, namely the number of molecules of the drug required to produce the same effect as that of one molecule of 5-hydroxytryptamine.

After many of the compounds, the rat uterus only returned to normal very slowly and the determination of these dose/response curves became very tedious, particularly with high doses. With such compounds, simple matching experiments were performed and the equipotent molar ratio was calculated by comparing the doses of 5-hydroxytryptamine and of the test drug which produced roughly 50% of the maximal contraction.

RESULTS

Effects on the Isolated Rat Uterus

The effects varied with the concentration as well as with the nature of the drug. Starting with those of low concentrations, they could be divided into: (1) Synergism with 5-hydroxytryptamine, tryptamine and acetylcholine, (2) antagonism of 5-hydroxytryptamine and tryptamine, (3) stimulant action, (4) production of repeated contractions, and (5) profound depression. These are illustrated in Fig. 1.

Synergism.—Many of the compounds showed synergism with 5-hydroxytryptamine, tryptamine and also with acetylcholine. This was observed when a dose of one of these was applied first alone, and then in the presence of a dose of the test drug which was too small to produce a visible effect by itself. The contraction which ensued was greater than that caused by the 5-hydroxytryptamine, tryptamine or acetylcholine alone.

This phenomenon was never observed with 5-benzyloxy-3-(2-dimethylaminoethyl)-indole (Table I, No. 15) or with 3-(2-dimethylaminoethyl)indole (No. 1) and was difficult to demonstrate with 3-(2-methylaminoethyl)indole (No. 9), 3-(2-dimethylaminoethyl)-2-methylindole (No. 12) and 3-(2-diethylaminoethyl)indole (No. 2). It was shown by all the other substances, but only over a narrow range of concentration. A 5-fold increase was usually adequate for the transition from no effect at all to stimulation of the preparation by the drug itself (action 3).

This type of experiment was designed to show any potentiation which might be caused by an action of the compounds in inhibiting the destruction of tryptamine or 5-hydroxytryptamine. By comparison with eserine and acetylcholine, for instance, such a potentiation might be expected to increase the effects of tryptamine or 5-hydroxytryptamine by a factor of at least ten or more, which is not what was observed here. The increase in the responses to tryptamine, 5-hydroxytryptamine and acetylcholine was noticeable, but such observations do not by themselves prove that the combined action of the two drugs was more than additive. It was possible to produce similar effects with 5-hydroxytryptamine itself (Fig. 2). When the dose/effect curve was steep it is not surprising that the response to a small dose should be increased by adding a paeneliminal dose of the same drug, or of some other stimulant drug. There was no evidence that these compounds had more effect than would be expected on this basis: all of them caused stimulation in slightly higher doses.

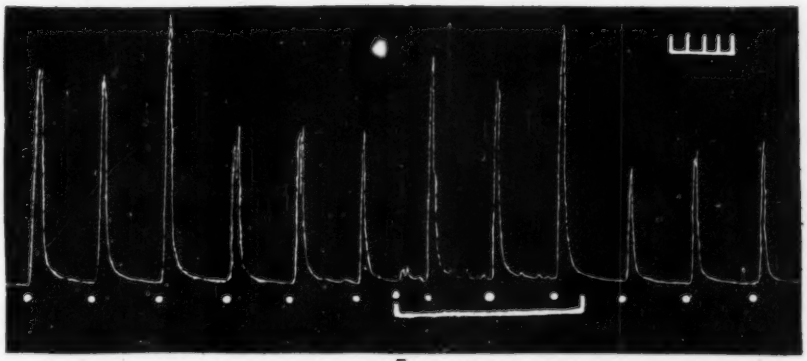

FIG. 2.—Isolated rat uterus preparation. Responses to 5-hydroxytryptamine (H), tryptamine (T) and acetylcholine (A) before, during, and after maintenance at F of a dose of 2·5 ng. of 5-hydroxytryptamine in the bath for the period indicated. This shows the synergism of all three drugs with the small background dose of 5-hydroxytryptamine. The numerals below H and A give the doses in ng., while those below T in μg. Time, min.

Antagonism of 5-Hydroxytryptamine and Tryptamine.—This was shown only by the compounds listed in Table II, which summarizes the results of quantitative experiments on the antagonistic properties of the compounds on this preparation. Gaddum, Hameed, Hathway, and Stephens (1955) obtained drug ratios of 0.66 for 5-benzyloxygramine (No. 16) and 0.027 for 3-(2-dimethylaminoethyl)indole (calculated on a molar basis), which agree quite well with our results. It is convenient to classify the compounds into antagonists comparable with lysergic acid diethylamide (which has a drug ratio of 37 on a molar basis), moderate antagonists such as 5-benzyloxygramine, 5-benzyloxy-3-(2-dimethylaminoethyl)indole and 3-(2-dimethylaminoethyl)-indole, and feeble antagonists, such as those shown in Table IIB. These latter have such weak activity that the dose ratio only was determined: the highest values of this were below the limit (about 5) at which it is possible to measure the drug ratio. In these experiments the acetylcholine controls were unaffected by the moderate antagonists, and tryptamine, in the few experiments performed with it, was antagonized in much the same way as 5-hydroxytryptamine. One of the feeble antagonists however, 3-(2-dimethylaminoethyl)-2-methylindole, increased the responses to acetylcholine and tryptamine slightly and, on this preparation, was quite a specific antagonist of 5-hydroxytryptamine.

TABLE II

ANTAGONISTIC ACTIVITY ON THE RAT UTERUS AT 36°

A—Drug Ratio Experiments using 5-Hydroxytryptamine. Note: The drug ratio is the ratio of the concentration of the agonist to the concentration of the antagonist when the response is 50% of the initial maximum. In this work, the antagonist was allowed to act for one hour: all concentrations are expressed in terms of molarity. The asterisk indicates that, at this high concentration, Gaddum, Hameed, Hathway and Stephens (1955) obtained similar high drug ratios which they ascribed to the development of an unsurmountable block.

Comp. No.	Drug	Conc. (M)	Drug Ratio (Mean±s.e.)	No. of Expts.
16	5-Benzyloxygramine ..	9.0×10^{-7} 18·0 *72·0	0·77 0·56 6·4	1 1 1
15	5-Benzyloxy-3-(2-dimethylaminoethyl)indole ..	90·0	0·066±0·008	4
1	3-(2-Dimethylaminoethyl)-indole	99·0	0·042±0·014	4

B—Dose Ratio Experiments. Note: The dose ratio is the number of times the concentration of the agonist must be increased in order to produce a 50% response after an antagonist has been allowed to act on the preparation for one hour. It should obviously increase with increasing the concentration of antagonist. The values for the dose ratio (below) and drug ratio (above in Table IIA) should not be confused. It is only possible to proceed to determine the drug ratio if a value above 5 can be obtained for the dose ratio. * Indicates potentiation: after washing this effect passed off much more quickly than did the antagonism of 5-hydroxytryptamine. † Indicates that the compound was allowed to act for 30 min. only. — Signifies not tested.

Comp. No.	Drug	Conc. (M)	Dose Ratios for: 5-Hydroxytryptamine	Tryptamine	Acetylcholine
1	3-(2-Dimethylaminoethyl)indole	1×10^{-6}	4 3·5	2·5 2	1 1
		3	7 5·5	2·5 1·5	1 1
		10	10	4	1
2	3-(2-Diethylaminoethyl)indole	4	4	—	1
12	3-(2-Dimethylaminoethyl)-2-methylindole	10	1·5 1·5 1	— <0·5* 0·25*	1 <0·5* 0·25*
9	3-(2-Methylaminoethyl)indole	12·5	3	—	1
7	3-(2-Piperidinoethyl)-indole	15†	2	—	1
8	3-(2-Morpholinoethyl)-indole	20†	2	1·5	1
18	3-(2-Aminobutyl)indole	42	4	—	1

Stimulant Action. — All the drugs except 5-benzyloxy-3-(2-dimethylaminoethyl)indole, 3-(2-aminobutyl)indole (No. 18) and 3-(2-morpholinoethyl)indole (No. 8) caused contraction of the rat uterus when given in suitable concentration. The equipotent molar ratios are shown in Table I. As with 5-hydroxytryptamine and tryptamine, there was a latent period of 5 to 10 sec. before the muscle started to respond, the contraction lasted 20 to 30 sec. and then passed off. 5-Hydroxytryptamine, tryptamine and 3-(2-aminopropyl)-indole were easily washed out and the preparation rapidly returned to normal, but the other compounds required more washing and a longer period for recovery. The dose/response curves for 5-hydroxytryptamine, tryptamine and 3-(2-aminopropyl)indole appeared to be parallel, as did those of the compounds whose agonist activity (Table I) was obtained from such curves. Of the other compounds, whose activity was estimated by matching (see above), all, with the sole exception of 3-(2-dimethylaminoethyl)indole, gave rise to maximal contractions when given in a large enough dose.

Production of Repeated Contractions.—When given in doses in excess of those which produced a maximal response, all the stimulant drugs caused repeated contractions of the preparation. Even though large doses of 3-(2-dimethylaminoethyl)indole failed to produce a maximal contraction they caused repeated (submaximal) responses. After treatment with 3-(2-dimethylaminoethyl)indole the preparation was noticeably

less sensitive to 5-hydroxytryptamine for a short time as it was also after similar treatment with 5-hydroxytryptamine, or with any of the drugs possessing antagonistic activity. After repeated contractions produced by some of the compounds which were purely stimulant, the tissue was more sensitive to 5-hydroxytryptamine for a short time.

Profound Depression.—In still higher concentrations, the preparation was quiet and insensitive to 5-hydroxytryptamine. The responses to acetylcholine were not always affected, but could be blocked by raising the concentration of the drug still further.

Effects on the Isolated Rat Fundus Strip Preparations

Modification of the Responses to 5-Hydroxytryptamine and Tryptamine by Very Low Concentrations.—When low concentrations of the drugs acted on the rat fundus strip, there did not appear to be any synergism with 5-hydroxytryptamine such as was found on the rat uterus. Some of the compounds enhanced the initial responses to tryptamine, and two substances, 3-(2-dimethylaminoethyl)indole and 3-(2-dimethylaminoethyl) - 2 - methylindole, antagonized 5 - hydroxytryptamine. The dose ratios at the end of 60 min. are shown in Table III. The 2-methyl compound, like some of the others, enhanced the responses to tryptamine in concentrations which antagonized 5 - hydroxytryptamine but 3 - (2 - dimethylaminoethyl)indole itself did not.

TABLE III

ANTAGONISTIC ACTIVITY ON THE RAT FUNDUS STRIP AT 37°

* Indicates potentiation.

Comp. No.	Drug	Conc. (M)	Dose Ratios for:	
			5-Hydroxytryptamine	Tryptamine
1	3-(2-Dimethylaminoethyl)indole	1×10^{-7}	2	1
		3·3	10	2
12	3-(2-Dimethylaminoethyl)-2-methylindole	20	10	0·5–1*
		20	5	0·5–1*

Stimulant Action.—In slightly higher concentrations, all the compounds stimulated the preparation, but some of them, particularly 5-benzyloxy-3-(2-dimethylaminoethyl)indole, 3-(2-aminopropyl)indole and 3-(2-dipropylaminoethyl)-indole (No. 3) produced a much slower contraction of the muscle than did 5-hydroxytryptamine or tryptamine. Recovery, also, was a much slower process. The equipotent molar ratios are shown in Table I.

The dose/response curves of all the compounds appeared to be parallel to those of 5-hydroxytryptamine and tryptamine, though there was some doubt about the results for 5-benzyloxy-3-(2-dimethylaminoethyl)indole because of its slow onset and long duration of action.

Depression.—After stimulation with these compounds, the preparation was insensitive to 5-hydroxytryptamine. When dose/response curves were being obtained it was particularly necessary to allow time for this depression to pass off and to wait until control responses to 5-hydroxytryptamine returned to normal before proceeding.

Differentiation Between the Actions of Tryptamine and 5-Hydroxytryptamine.—In the course of testing these compounds it became apparent that there were differences between the antagonism of tryptamine and the antagonism of 5-hydroxytryptamine. Although, on both the rat uterus and the rat fundus strip, the dose/response curves for tryptamine and 5-hydroxytryptamine were roughly parallel, the existence of substances which antagonized one rather than the other made it seem likely that tryptamine and 5-hydroxytryptamine were not acting in the same way or at the same site. It was therefore necessary to see whether the stimulant action of our substances was tryptamine-like or 5-hydroxytryptamine-like.

In spite of the fact that some of the substances tested, particularly 3-(2-dimethylaminoethyl-2-methyl)indole, were fairly specific in their antagonism of 5-hydroxytryptamine, they could not be used for analysing the stimulant action of the drugs on the rat fundus strip because they were themselves stimulant in higher concentrations. Woolley and Shaw (1957) found that 1-benzyl-3-(2-dimethylaminoethyl)-5 - methoxy - 2 - methylindole (BAS) was much more effective as an antagonist of 5-hydroxytryptamine than as an antagonist of tryptamine when tested on the blood pressure of the anaesthetized dog. Table IV shows that, on the rat fundus strip, it had relatively little antagonistic activity, whereas 2-bromolysergic acid diethylamide was suitable for studying specifically the antagonism of 5-hydroxytryptamine. Concentrations of this compound which affected the responses to tryptamine only slightly had a pronounced effect on the responses to 5-hydroxytryptamine and (unlike similar concentrations of lysergic acid diethylamide) did not themselves cause any contraction or even disturb the base-line. It was, however, found, in the presence of 2-bromolysergic acid diethylamide, that the contraction of the muscle took longer to develop, even in response to 5-hydroxytryptamine

TABLE IV

ANTAGONISM OF 5-HYDROXYTRYPTAMINE, TRYPTAMINE, 3-(2-DIMETHYLAMINOETHYL)INDOLE AND 3-(2-DIPROPYLAMINOETHYL)INDOLE ON THE RAT FUNDUS STRIP AT 37°

The antagonistic effect required 30 to 40 min. to develop completely: this period was allowed for the action of BAS, but in the experiments with Brom-LSD this antagonist was allowed to act for 60 min. BAS = 1-Benzyl-3-(2-dimethylaminoethyl)-5-methoxy-2-methylindole (Woolley and Shaw, 1957). Brom-LSD = 2-Bromolysergic acid diethylamide. 5-HT = 5-Hydroxytryptamine. Me_2N = 3-(2-Dimethylaminoethyl)indole (No. 1). nPr_2N = 3-(2-Dipropylaminoethyl)indole (No. 3).

		Dose Ratios with			
		5-HT	Me_2N	nPr_2N	Tryptamine
BAS					
$3{\cdot}1\times10^{-7}$ M	..	1	—	—	1
$3{\cdot}1\times10^{-6}$	..	2	—	—	1
$7{\cdot}8\times10^{-6}$	..	4	—	—	1
Brom-LSD					
5×10^{-9} M	..	1·5	—	—	1
$1{\cdot}2\times10^{-7}$	(i)	40	—	—	4
	(ii)	50	40	—	7
	(iii)	15	—	15	—
	(iv)	5	>10	—	2
	(v)	10	—	10	5
$2{\cdot}5\times10^{-7}$	..	20	—	—	7
$3{\cdot}7\times10^{-7}$	(i)	40	—	40	10
	(ii)	25	53	—	6
$7{\cdot}5\times10^{-7}$	..	100	—	—	10
$2{\cdot}5\times10^{-6}$	(i)	130	—	—	>10
	(ii)	>200	—	—	6

or tryptamine, and in these experiments a period of up to 3 min. was allowed (instead of 90 sec.) after the addition of the stimulant drugs.

The contractions produced by 3-(2-dimethylaminoethyl)indole and 3-(2-dipropylaminoethyl)-indole were depressed in much the same way as those produced by 5-hydroxytryptamine, the dose ratios being quite similar to those of 5-hydroxytryptamine and different from those of tryptamine. Another interesting fact which emerges from Table IV is that, above a certain level (roughly 2×10^{-7} M), increasing the concentration of 2-bromolysergic acid diethylamide by as much as a factor of 10 did not increase the block of tryptamine, although it greatly increased the block of 5-hydroxytryptamine.

DISCUSSION

If synergism on the rat uterus is accepted as a simple addition of two actions, the results from the two preparations are not dissimilar. Most of the compounds were purely stimulant, but some combined an antagonism of 5-hydroxytryptamine (and, to varying extents, of tryptamine) with a stimulant action at higher concentrations. 5-Benzyloxygramine and 5-benzyloxy-3-(2-dimethylaminoethyl)indole alone were purely antagonists on the rat uterus, but even they could cause stimulation of the rat fundus strip.

Antagonistic activity is confined to relatively few of the compounds; the most active are those with the dimethylamino group in the side-chain. In contrast to the results of Gaddum, Hameed, Hathway, and Stephens (1955) and of Woolley and Shaw (1953), introduction of a methyl group in the 2 position of the indole nucleus of these simple analogues of tryptamine did not increase the antagonistic activity although it did seem to endow it with a certain degree of specificity for 5-hydroxytryptamine. Another unexpected discovery was that 5-benzyloxy-3-(2-dimethylaminoethyl)indole was much less active than 5-benzyloxygramine. It was anticipated that the former, being a substituted tryptamine, would be more active than the latter, which has only one methylene group in the side-chain. One of the most interesting results was the existence among compounds tested of differences between antagonism of 5-hydroxytryptamine and tryptamine. It is odd that these tryptamines without a 5-hydroxyl group, such as 3-(2-dimethylaminoethyl)-2-methylindole, should have antagonized 5-hydroxytryptamine much more effectively than they antagonized tryptamine.

Stimulant activity was shown by many of the compounds and seemed to vary in quite a definite manner with chemical structure. It is disappointing, though probably significant, that the high activity of 3-(2-dipropylaminoethyl)indole on the rat fundus strip was not so marked on the rat uterus. It is not clear whether this compound is the most active member of the 3-(2-dialkylaminoethyl)indoles because the propyl group is specifically associated with activity or simply because the size of that part of the molecule has reached a critical limit. It would be interesting to know if the activity of the 2-methyl substituted compounds is also maximal in the dipropylamino member of the series. It is astonishing that, although these compounds lack a 5-hydroxyl group, their stimulant activity appears to resemble that of 5-hydroxytryptamine rather than that of tryptamine, and so, both in antagonistic and stimulant properties, the drugs influenced the actions of 5-hydroxytryptamine rather than those of tryptamine. Another important finding was that those of the compounds which have antagonistic activity cause stimulation in concentrations greater than those which cause antagonism: this combination of antagonistic and stimulant properties is the reverse of what would be expected if the compounds were partial agonists (Stephenson,

1956). The present evidence does not justify extensive speculation about the modes of action of 5-hydroxytryptamine and tryptamine. This problem is being studied further, and it must be better understood before any attempt is made to discuss relationships between chemical structure and pharmacological properties either antagonistic or stimulant. It is possible, for instance, that the anomalously high activity of 5-benzyloxygramine compared with 5-benzyloxy-3-(2-dimethylaminoethyl)indole may be due to some differences between the modes of action of the two. It is likewise tempting to look for a difference between the actions of really powerful antagonists, such as 2-bromolysergic acid diethylamide, and the moderate antagonists described here, which have a greater formal resemblance to tryptamine and 5-hydroxytryptamine.

We wish to express our most sincere thanks to Professor J. H. Gaddum, who suggested the problem to us and who has given us much helpful advice, to Dr. J. R. Vane and Mr. R. P. Stephenson, with whom we have had many useful discussions, and to Dr. Vane particularly for initiating us into the use of the rat fundus strip. This work was done during the tenure by one of us (I. K.) of a Pakistan Government Scholarship.

The specimens of 3-(2-aminopropyl)indole and 3-(2-aminobutyl)indole hydrochlorides were kindly sent us by Dr. H. C. Carrington of Imperial Chemical (Pharmaceuticals) Ltd. We thank Dr. M. E. Speeter, The Upjohn Company, for the gift of 5-hydroxytryptamine creatinine sulphate, Dr. D. W. Woolley, The Rockefeller Institute, New York, for 1-benzyl-3-(2 - dimethylaminoethyl) - 5 - methoxy - 2 - methyl - indole, and Sandoz A. G. for 2-bromolysergic acid diethylamide.

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THE EFFECTS OF RESERPINE AND OTHER AGENTS UPON LEPTAZOL CONVULSIONS IN MICE

BY

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Reserpine and tetrabenazine reduced the survival time of mice infused with leptazol. This effect was antagonized by pretreatment with iproniazid. The survival time of iproniazid-treated mice was prolonged by 5-hydroxytryptophan but not by 3:4-dihydroxyphenylalanine. These findings suggest a relation between leptazol sensitivity and brain levels of 5-hydroxytryptamine.

It has been shown by Chen and his co-workers (Chen, Ensor, and Bohner, 1954) that the survival time of mice infused intravenously with leptazol may be shortened by pretreatment with reserpine. Reserpine is unique in being the only sedative agent for which this property has been shown; barbiturates lengthen survival time (Chen *et al.*, 1954), while chlorpromazine has been reported to be without effect (Balestrieri, 1955). The sedative properties of reserpine have been attributed to the changes which this drug produces in brain levels of 5-hydroxytryptamine and catechol amines. We have therefore investigated the action of drugs altering brain amine metabolism and other agents upon the facilitation of leptazol by reserpine. After this work was completed, a paper appeared describing experiments similar to those reported below and reaching essentially the same conclusions (Kobinger, 1958).

METHOD

The method used by Chen *et al.* (1954) was that due to Orloff, Williams and Pfeiffer (1949). They injected mice intravenously with 0.05 ml. of a solution containing 0.25 mg. of leptazol every 10 sec. The volume injected between the first twitch and the terminal extensor spasm was the measure found to be reduced by reserpine; the volume injected before the first sign appeared was unaffected.

We have used a constant-rate injection machine for infusing into the tail vein a 0.5% leptazol solution containing a dye at the rate of 0.24 ml./min. LAC grey mice of either sex were used, weighing 16 to 23 g. We have preferred to measure the total period from commencing the infusion until the terminal convulsion occurred and this we have called the survival time. Drugs were injected intraperitoneally in volumes of 0.1 ml./10 g. of body weight.

RESULTS

The mean survival time of 139 mice infused with leptazol was $114.1 \pm$ s.e. 2.8 sec. This value is derived from animals used, in groups of about 10, as controls in a number of experiments. The mean values for these groups ranged from 90 to 130 sec. with standard errors from ± 7 to ± 15 sec.

Rauwolfia Alkaloids

Reserpine.—The change in survival time of groups of mice infused with leptazol at various intervals after treatment with 2 mg./kg. of reserpine is shown in Fig. 1. It will be seen that no significant reduction occurred up to 1¼ hr. after reserpine ($P>0.05$), but that from 1¾ hr. survival time became shorter ($P<0.01$), reaching a minimum in about 4 hr. and was still slightly below normal 3 days after reserpine ($P<0.05$). The dose of reserpine used produced hypothermia and sedation in mice, lasting about 2 days. Their body temperatures measured at the end of this period, by a method previously described (Lessin and Parkes, 1957), had regained normal values ($37.2 \pm 0.2°$).

With doses of reserpine smaller than 2 mg./kg., no significant effect upon survival time could be shown.

Tetrabenazine

This compound, 3-*iso*butyl-1:2:3:4:6:7-hexahydro-9:10-dimethoxy-11bH-benzo[*a*]quinolizin-2-one (Pletscher, 1957a; Pletscher, Besendorf, and Bächtold, 1958), is a member of a group of synthetic compounds shown to reduce brain amine levels. It causes sedative effects in

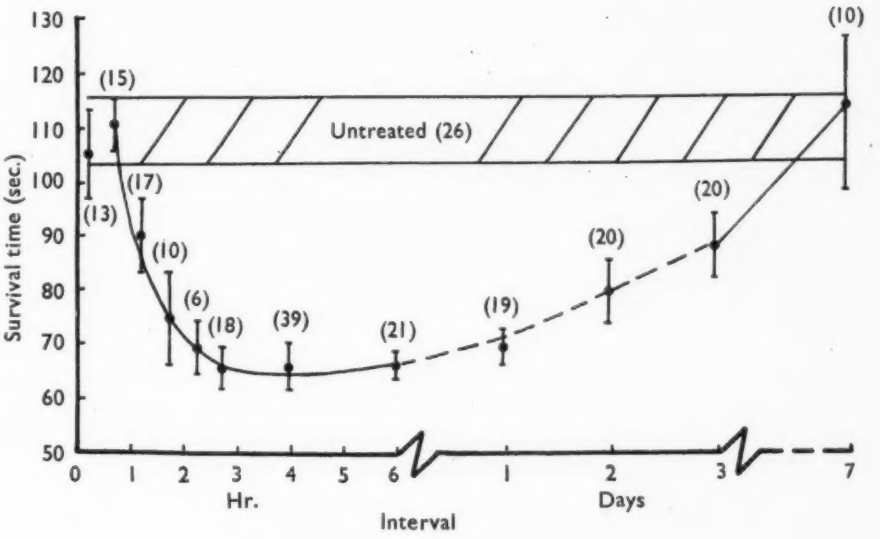

FIG. 1.—Leptazol survival time of groups of mice pretreated with 2 mg./kg. of reserpine intraperitoneally. Ordinate, survival time in sec. Abscissa, interval between reserpine injection and leptazol infusion. Each point is the mean for the group, with its standard error; the number of mice in the group is shown in brackets. The shaded area gives the limits between the mean value ± s.e. for survival time of mice untreated with reserpine.

experimental animals resembling those of reserpine, although of shorter duration. Table I shows that this compound (75 mg./kg.) caused considerable reduction of survival time within 1½ hr.

Agents Affecting Amine Metabolism

Iproniazid.—Pretreatment with 200 mg./kg. of iproniazid did not affect the survival time of mice infused with leptazol 3 to 24 hr. later (Table I), but this treatment prevented the reduction of survival time by effective doses of reserpine injected 24 hr. later. Hertting (1958) has also demonstrated this antagonism. Tetrabenazine was also rendered ineffective by pretreatment with 200 mg./kg. of iproniazid (Table I). This dose of iproniazid is adequate for complete inhibition of brain monoamine oxidase in mice within 24 hr. (Davison, Lessin, and Parkes, 1957).

(±)-5-*Hydroxytryptophan.*—This agent, in doses of 50 mg./kg. injected 1 hr. before leptazol infusion, lengthened the survival time of mice that had been treated with iproniazid (200 mg./kg.) 24 hr. previously (Table I). When this pretreatment was omitted, 5-hydroxytryptophan did not alter survival time, even in a dose of 100 mg./kg., nor did it produce the tremors observed when 5-hydroxytryptophan was administered to iproniazid-treated mice.

(±)-3:4-*Dihydroxyphenylalanine.*—This substance (50 mg./kg.) given 45 min. before leptazol infusion produced no alteration in survival time of mice pretreated with iproniazid (Table I). Signs of excitement were observed in

TABLE I

EFFECT OF VARIOUS DRUGS UPON THE SURVIVAL TIME OF MICE INFUSED INTRAVENOUSLY WITH LEPTAZOL

The numerals in brackets give the number of animals used.

Treatment	Interval between Last Treatment and Leptazol	Mean Survival Time (Sec. ± s.e.)	
Iproniazid, 200 mg./kg.	3 hr.	106·0 ± 16·2 (6)	$P=0·5$
Control	—	122·6 ± 11·7 (7)	
Reserpine, 2 mg./kg.	2 hr.	74·0 ± 9·0 (10)	$P<0·01$
Reserpine, 2 mg./kg., 24 hr. after iproniazid, 200 mg./kg.	2 ,,	117·3 ± 15·4 (6)	$P<0·05$
Tetrabenazine, 75 mg./kg.	1½ hr.	44·1 ± 2·2 (11)	$P=0·001$
Control	—	94·4 ± 12·4 (10)	
Iproniazid, 200 mg./kg.	24 hr.	117·7 ± 11·8 (10)	$P=0·5$
Tetrabenazine, 75 mg./kg., 24 hr. after iproniazid, 200 mg./kg.	1½ ,,	108·1 ± 10·6 (12)	
5-hydroxytryptophan, 50 mg./kg.	30 min.	103·8 ± 10·0 (10)	$P=0·7$
Control	—	98·0 ± 8·7 (10)	
Iproniazid, 200 mg./kg.	24 hr.	95·9 ± 7·3 (10)	$P<0·001$
5-Hydroxytryptophan, 50 mg./kg., 24 hr. after iproniazid, 200 mg./kg.	30 min.	148·7 ± 6·6 (12)	
Dihydroxyphenylalanine, 100 mg./kg., 24 hr. after iproniazid, 200 mg./kg.	30 min.	93·1 ± 11·6 (9)	
Control	—	106·5 ± 6·0 (8)	
Lysergic acid diethylamide, 2·5 mg./kg.	15 min.	119·7 ± 8·4 (12)	
Reserpine, 2 mg./kg.	4½ hr.	65·3 ± 7·1 (17)	$P<0·01$
Lysergic acid diethylamide, 2·5 mg./kg., 4 hr. after reserpine, 2 mg./kg.	15 min.	93·2 ± 5·8 (12)	
Reserpine, 2 mg./kg.	4½ hr.	55·9 ± 1·0 (8)	P 0·1–0·2
2-Bromo-(+)-lysergic acid diethylamide, 2·5 mg./kg., 4 hr. after reserpine, 2 mg./kg.	15 min.	68·7 ± 8·6 (12)	

these mice following the injection of dihydroxyphenylalanine similar to those reported by Carlsson, Lindquist, and Magnusson (1957).

Other Agents

(+)-*Lysergic Acid Diethylamide.* — Lysergic acid diethylamide (2.5 mg./kg.) antagonized the effect of reserpine on leptazol survival time when given 5 to 20 min. before the convulsant. This dose did not affect the survival time of normal mice (Table I).

2-Bromo-(+)-lysergic Acid Diethylamide

This compound shares with lysergic acid diethylamide the property of antagonizing the stimulant actions of 5-hydroxytryptamine upon smooth muscle, while lacking the centrally excitant properties of the parent compound (Cerletti and Rothlin, 1955). It was without action on the survival time of mice treated with reserpine under the same experimental conditions as those in which lysergic acid diethylamide showed antagonism to reserpine facilitation (Table I).

Discussion

Comparison of the time course of facilitation of leptazol convulsions with that of sedation and hypothermia by similar doses of reserpine in mice (Lessin and Parkes, 1957) showed that, whereas both types of effect were delayed in appearance for an hour or so after administration and reached their greatest intensity over the period 2 to 24 hr., they differed in duration. Survival time had not regained normal levels 3 days after reserpine, although body temperature had recovered within 2 days. A similar comparison may be drawn between the time course of leptazol facilitation by reserpine and the reduction in the levels of brain 5-hydroxytryptamine which this drug produced. This effect commenced without delay and was maximal 4 hr. after injection, while only partial recovery has occurred by 2 days in the mouse (Pletscher, personal communication) as in the rabbit (Pletscher, Shore, and Brodie, 1956). The similarity between the time courses of these two effects suggests that a relation may exist between leptazol facilitation and brain amine level similar to that postulated for the sedative properties of reserpine (Pletscher, Shore, and Brodie, 1956).

Further evidence for this relationship is provided by the action of iproniazid, since this agent, which has been shown to prevent the depletion of brain 5-hydroxytryptamine by reserpine (Besendorf and Pletscher, 1956; Pletscher, 1956), could also abolish the effect of reserpine upon leptazol survival time. In addition, the action of tetrabenazine, which both facilitated leptazol convulsions and reduced brain 5-hydroxytryptamine (Pletscher, 1957b ; Pletscher, Besendorf, and Bächtold, 1958), effects which are prevented by iproniazid, lends further support to the argument.

Although treatment with iproniazid alone had no effect upon survival time, the subsequent administration of 5-hydroxytryptophan increased resistance to leptazol. This combination has been shown to raise brain levels of 5-hydroxytryptamine considerably (Udenfriend, Weissbach, and Bogdanski, 1957) and this may be seen as an extension of the relationship between sensitivity to leptazol and brain 5-hydroxytryptamine level. We were unable to demonstrate the protecting action of dihydroxyphenylalanine in iproniazid-treated mice reported by Kobinger (1958). Since administration of dihydroxyphenylalanine after iproniazid raised the level of brain catechol amines (Pletscher, 1957b), it may be suggested that these amines do not play a part in leptazol sensitivity. This contrasts with the situation regarding the sedative actions of reserpine, for which Carlsson *et al.* (1957) found dihydroxyphenylalanine a better antagonist than 5-hydroxytryptophan.

The action of lysergic acid diethylamide in antagonizing the facilitating effect of reserpine is likely to be of a different character from that of the agents just discussed. The interaction of lysergic acid diethylamide with 5-hydroxytryptamine is that of an antagonist, and this property is unlikely to play a part in its effect upon leptazol facilitation, since the similarly active compound 2-bromo-(+)-lysergic acid diethylamide was without effect. It is more reasonable to suppose that a central stimulant action of lysergic acid diethylamide is involved, since 2-bromo-(+)-lysergic acid diethylamide lacks such action, but it is, apparently, not an activity which has any effect upon the threshold of normal mice to leptazol.

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MECHANISM OF ACTION OF RESERPINE IN PRODUCING GASTRIC HAEMORRHAGE AND EROSION IN THE MOUSE

BY

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Gastric haemorrhage was produced regularly in mice within 6 hours of the subcutaneous injection of a large dose (2 to 10 mg./kg.) of reserpine or of deserpidine. Rescinnamine, syrosingopine (SU-3118), and tetrabenazine (Ro 1-9569) were less active. Gastric haemorrhage was also produced within 6 hours when 5-hydroxytryptamine (10 mg./kg.) was injected every half-hour. Neither reserpine nor 5-hydroxytryptamine produced gastric haemorrhage in mice which had been vagotomized by tying the oesophagus at the cardio-oesophageal junction or which had been pre-treated with iproniazid. Amphetamine was less effective than iproniazid in preventing gastric haemorrhage after reserpine, and the following drugs were ineffective: cocaine, methyl phenidate (Ritalin), amarin, caffeine, nikethamide, lysergic acid diethylamide and its 2-bromo derivative (BOL148). Gastric haemorrhage was not observed in mice which had been given substantial doses of atropine or of hexamethonium before reserpine. The incidence of haemorrhage was substantially reduced by treatment with an antacid mixture. It is concluded that reserpine-like drugs cause gastric haemorrhage by a mechanism which has an important central component and which involves the liberation of 5-hydroxytryptamine.

Haverback and Bogdanski (1957) have shown that gastric erosion was produced in rats within 18 hr. of the injection of large doses of 5-hydroxytryptophan. Reserpine in large doses had a similar effect (Benditt and Wong, 1957; Haverback and Bogdanski, 1957; Blackman, Campion, and Fastier, 1958). Some of the pharmacological actions of both these drugs appear to be mediated by 5-hydroxytryptamine. Reserpine liberates 5-hydroxytryptamine from platelets and certain other parts of the body, and interferes with the uptake of 5-hydroxytryptamine, while 5-hydroxytryptophan is the natural precursor to 5-hydroxytryptamine (Pletscher, Shore, and Brodie, 1955; Page, 1958). Therefore, when we observed in experiments on mice that large doses of reserpine produced gastric haemorrhage and melaena within a few hours (Blackman *et al.*, 1958), we supposed at first that this was a peripheral effect brought about by an increase in "free" 5-hydroxytryptamine. The possibility that it was a central effect was raised by our observation that reserpine did not produce gastric haemorrhage in mice which had been vagotomized at the cardio-oesophageal junction. Another observation at variance with our original hypothesis was that reserpine did not produce gastric haemorrhage in mice which had been previously treated with iproniazid (Blackman *et al.*, 1958). Since iproniazid is a powerful inhibitor of mono-amine oxidase and is believed to delay the destruction of 5-hydroxytryptamine in the body (Page, 1958), it would be expected to enhance rather than antagonize the harmful effect of reserpine on the gastric mucosa, were the erosion brought about by the local action of 5-hydroxytryptamine. These anomalous findings induced us to carry out the experiments reported here.

METHODS

Male mice weighing 30 to 40 g. were used. During the 24 hr. period preceding an experiment, they were fed only on milk and water. They were killed at the end of an experiment by breaking their necks. The abdomen was then opened and the stomach examined for the presence of blood and the intestine for melaena. Doubtful results were recorded as negative.

Vagotomy was performed under ether anaesthesia. When the vagi were cut in the neck, adverse respiratory effects were usually obtained not long afterwards. It was therefore thought preferable to

vagotomize by ligating the oesophagus as close to the diaphragm as was possible. This procedure was always used in these experiments because we found that separating the vagi from the oesophagus was impracticable. Control groups of mice were subjected to a mock operation in which the abdomen was opened and the intestines were handled to the same extent as in the vagotomized animals.

For the estimation of gastric hydrochloric acid, mice were anaesthetized with ether. The abdomen was opened and the oesophagus tied just above the cardiac sphincter (if this had not already been done in the course of vagotomy). Next, a cannula was inserted into the first part of the duodenum, pushed through the pyloric sphincter into the stomach, and tied in position. The stomach contents were washed out with about 10 ml. of distilled water by means of a syringe fitted with a fine-gauge needle which was pushed through the stomach wall. The stomach washings were filtered. After the filtrate had been made up to a volume of 100 ml., 25 ml. portions of this were titrated against 0.01 N-NaOH using phenolphthalein as an indicator.

The antacid used was usually an aluminium hydroxide gel (Amphogel, Wyeth) diluted with distilled water to the extent specified in the text. The antacid solution was injected into the lower oesophagus by means of a fine lubricated polythene tube. The mice of control groups were given distilled water in equal volume by this route.

All the drugs used except amarin were obtained from commercial sources. Reserpine and related compounds were brought into solution with the aid of ascorbic acid (up to 1%).

RESULTS

Gastric Haemorrhage after Reserpine

Reserpine given subcutaneously in a dose of 10 mg./kg. almost invariably caused gross gastric haemorrhage within 6 hr. In 8 successive experiments, only 2 out of 45 mice failed to show gastric haemorrhage. Multiple erosions with bleeding vessels were observed often. Melaena was seen in most mice. Haemorrhage was observed in some mice killed within 30 min. of treatment with reserpine. The incidence of gastric haemorrhage remained high when the dose of reserpine was reduced to 2.5 mg./kg. (Table I). With both doses of reserpine the mice were sedated within 30 min.; they had marked ptosis and some piloerection; and they appeared to suffer abdominal discomfort, some mice having diarrhoea.

TABLE I

INCIDENCE OF GASTRIC HAEMORRHAGE IN GROUPS OF 5 MICE 18 HR. AFTER THE SUBCUTANEOUS INJECTION OF RESERPINE AND RELATED COMPOUNDS

Sedation: ++, marked; + slight; − none.

Drug	2·5 mg./kg.		10·0 mg./kg.	
	Degree of Sedation	Incidence of Haemorrhage	Degree of Sedation	Incidence of Haemorrhage
Reserpine ..	++	5/5	++	5/5
Deserpidine ..	++	5/5	++	5/5
Rescinnamine ..	−	0/5	++	4/5
Syrosingopine ..	−	0/5	+	3/5
Tetrabenazine ..	−	1/5	+	1/5

Effect of Vagotomy by Tying the Oesophagus.—Mice which had been vagotomized at the cardio-oesophageal junction and another group upon which a mock vagotomy had been performed were given reserpine (10 mg./kg.) subcutaneously. Both groups of mice were sedated and had ptosis and piloerection within 30 min. of the injection of reserpine. Most mice showed signs of abdominal discomfort. Nevertheless, whereas gastric haemorrhage was obtained in 5 out of 6 control mice, it was not obtained in any of the 6 vagotomized mice (Table II).

TABLE II

EFFECTS OF VARIOUS TREATMENTS ON INCIDENCE OF GASTRIC HAEMORRHAGE 6 HR. AFTER THE ADMINISTRATION OF RESERPINE OR OF 5-HYDROXYTRYPTAMINE TO MICE

The first of each set of results gives the number of mice out of a group of 6 showing gastric haemorrhage. Reserpine was given subcutaneously in a single dose; other drugs were given in divided doses. Atropine sulphate (total 12 mg./kg.), hexamethonium bromide (total 120 mg./kg.), and 5-hydroxytryptamine (5-HT) (total 120 mg./kg.) were each given in a series of 12 doses at 30 min. intervals. Iproniazid was given in 2 doses each of 100 mg./kg., the first 24 hr. before and the second 30 min. before the injection of reserpine.

Treatment		Reserpine		5-HT	
	Dose (mg./kg.)	Treated	Control	Treated	Control
Vagotomy ..	—	0/6	5/6	1/6	5/6
Atropine	12 × 1	1/6	6/6	—	—
Hexamethonium	12 × 10	0/6	6/6	—	—
Iproniazid ..	2 × 100	0/6	6/6	1/6	5/6

Effect of Atropine and Hexamethonium.—These drugs were given to produce a "chemical" vagotomy. Each was injected subcutaneously 30 min. before the reserpine was given and then at 30 min. intervals during the 6 hr. that the mice were under the influence of reserpine. As may be seen from Table II, both drugs were highly effective in preventing gastric haemorrhage after reserpine.

Influence of Gastric Acidity.—The possibility that vagotomy could prevent the occurrence of gastric haemorrhage after reserpine by diminishing the secretion of acid was next considered. It was found that the free acid content of the stomachs of mice which had been vagotomized before treatment with reserpine was actually greater than that of normal mice treated

with reserpine. A second experiment gave substantially the same results as those summarized in Table III.

TABLE III

GASTRIC ACID CONTENT AND INCIDENCE OF GASTRIC HAEMORRHAGE IN MICE 2 HR. AFTER RESERPINE

Reserpine (10 mg./kg.) was injected subcutaneously. Those mice which had been vagotomized were allowed to recover fully from ether anaesthesia before they were given reserpine.

Treatment	Mean HCl 10^{-5}M (with s.e.)	Incidence of Haemorrhage
—	1·5 (±0·2)	0/5
Reserpine	1·8 (±0·4)	3/5
,, +vagotomy ..	3·5 (±0·9)	0/5

To see whether the incidence of gastric haemorrhage after reserpine could be reduced by diminishing gastric acidity, experiments were performed in which antacids were introduced into the stomach. Only 2 out of 6 mice which had received at 45 min. intervals eight 0.1 ml. doses of amphogel diluted 1:5 with water showed gastric haemorrhage when killed 6 hr. after the injection of reserpine (5 mg./kg.), whereas all the mice of the control group did. In another experiment in which a stronger solution of the antacid was used (amphogel diluted 1:2 with distilled water), none of the treated mice showed gastric haemorrhage. In this experiment, however, the reserpine was less effective than usual, only 2 of the 5 controls showing haemorrhage. Four 0.1 ml. doses of a dilute (1:10) amphogel solution gave no protection, the incidence of haemorrhage being 6 out of 6 for both the mice which had been given amphogel and for those which had been given saline. For another group of mice which had been treated at the same time with sodium bicarbonate (given in hourly doses of 0.1 ml. of a 0.5% solution) the incidence of haemorrhage was 4 out of 6.

Gastric Haemorrhage after other Drugs

Reserpine-like Drugs.—Two of the other compounds tested, deserpidine and rescinnamine, are also *Rauwolfia* alkaloids. Syrosingopine (SU - 3118; methyl *O* - 4 - ethoxycarbonyl - 3:5 - dimethoxybenzoylreserpate) is a semi-synthetic compound closely related to reserpine. Tetrabenazine (Ro 1-9569; 3-*iso*butyl-1:2:3:4:6:7-hexahydro - 9:10 - dimethoxy - 11bH - benzo[*a*] - quinolizine-2-one) is not at all closely related to reserpine chemically but resembles it pharmacologically (Pletscher, 1957).

When given in the same quantities as reserpine, deserpidine showed comparable activity (Table I). The other three compounds were less active, not only in producing gastric haemorrhage, but also in producing sedation.

5-Hydroxytryptamine.—When given to mice in large divided doses (10 mg./kg. subcutaneously every 30 min.), 5-hydroxytryptamine caused severe gastric haemorrhage in 4 out of 6 mice examined 3 hr. later and in 5 out of 6 mice examined 6 hr. later. The mice were not obviously sedated at any stage of the experiment. Other experiments with 5-hydroxytryptamine were performed on mice which had been subjected to either a real or a mock vagotomy. After 6 hr. treatment with 5-hydroxytryptamine (given in 12 doses each of 10 mg./kg. at 30 min. intervals), gastric haemorrhage was observed in only 1 out of 6 vagotomized mice but in 5 out of 6 control mice (Table II).

Pilocarpine.—The effect of vagotomy in the experiments just described suggested that increased vagal tone might play an important part in the production of gastric haemorrhage. The effect of a parasympathomimetic drug, pilocarpine, was therefore studied in both normal and vagotomized mice. Pilocarpine was given subcutaneously in 5 divided doses, each of 10 mg./kg., over a period of 4 hr. The amount given was comparable with that used by Underhill and Freiheit (1928) for the production of gastric erosion in rabbits. Of the 6 normal mice so treated, 5 showed gastric haemorrhage. All 6 vagotomized mice showed gastric haemorrhage and melaena.

Effect of Iproniazid

Previously (Blackman *et al.*, 1958) we performed some experiments with iproniazid at a time when we had little 5-hydroxytryptamine, and thought that we could enhance the effect of a moderate dose by treatment with an amine oxidase inhibitor. We found that gastric haemorrhage was not produced in mice which had been given large doses of iproniazid before treatment with 5-hydroxytryptamine was begun; nor was haemorrhage produced in mice which had been given iproniazid before reserpine (Table II). This made us wonder whether the gastric erosion produced by reserpine could be prevented by treatment with drugs of such types as central stimulants and amine oxidase inhibitors. Experiments were therefore performed with the substances listed in Table IV.

In the doses which we employed, only one of these substances reduced the incidence of gastric haemorrhage after reserpine, namely amphetamine. The central depressant action of

TABLE IV

EFFECTS OF VARIOUS TREATMENTS ON THE INCIDENCE OF GASTRIC HAEMORRHAGE 6 HR. AFTER THE ADMINISTRATION OF RESERPINE

All mice were given reserpine (2 mg./kg.) subcutaneously. Marked sedation is indicated as + +, mild sedation (lasting about an hour) by + and − indicates no sedation. The other drugs mentioned were given by subcutaneous injection usually in divided doses. For example, caffeine was given in 3 doses, each of 15 mg./kg., at 2 hr. intervals. LSD25=lysergic acid diethylamide and BOL148 is the 2-bromo derivative of this compound.

Treatment	Dose (mg./kg.)	Degree of Sedation	Incidence of Haemorrhage	
			Treated	Untreated
Iproniazid	2 × 100	−	0/6	6/6
Amphetamine ..	3 × 2·5	−	1/6	5/6
LSD25	5 × 3	−	6/6	6/6
Cocaine	2 × 7·5	+	6/6	3/6
Caffeine	3 × 15	+	6/6	5/6
Methyl phenidate ..	4 × 100	+	6/6	6/6
Nikethamide ..	5 × 100	+ +	5/6	6/6
Amarin	1 × 10	+ +	6/6	6/6
BOL148	5 × 10	+ +	6/6	6/6

reserpine was antagonized fairly effectively by amphetamine in the dose used (which was sufficiently large to kill some of the mice). Lysergic acid diethylamide antagonized the sedative action of reserpine even more effectively than did amphetamine; mice previously treated with lysergic acid diethylamide appeared to be hyperactive. However, these mice showed worse gastric haemorrhage than did mice given reserpine alone. Lysergic acid diethylamide was given in large divided doses because it is known to be rapidly metabolized in mice. 2-Bromolysergic acid diethylamide had no obvious effect on the response to reserpine. The central depressant action of reserpine was not antagonized strongly by caffeine, cocaine, or methyl phenidate (Ritalin) and was hardly affected by amarin or nikethamide.

DISCUSSION

Considerable evidence has been obtained in support of the hypothesis, proposed first by Brodie and his co-workers, that the pharmacological activity of reserpine is due in large measure to its ability to increase the amount of "free" 5-hydroxytryptamine present in the body (Page, 1958). The possibility must therefore be considered that reserpine in large doses produces gastric haemorrhage and erosion in the mouse and the rat by releasing 5-hydroxytryptamine from physiologically inert combinations in amounts sufficient to have a local deleterious effect.

That this action of reserpine is mediated by 5-hydroxytryptamine is strongly suggested by our finding that 5-hydroxytryptamine itself, if it is given repeatedly in large doses, produces gastric haemorrhage in the mouse. Other workers (Wilhelmi, 1957; Hedinger and Veraguth, 1957) have shown that 5-hydroxytryptamine in large doses causes gastric ulceration in the rat. The 5-hydroxytryptamine precursor, 5-hydroxytryptophan, has also been shown to produce gastric mucosal erosion in the rat (Haverback and Bogdanski, 1957).

Haverback and Bogdanski (1957) observed that lesions after the administration of 5-hydroxytryptophan were fewer or even absent if the rats had been atropinized. We have found similarly that the incidence of gastric haemorrhage after reserpine is greatly reduced by previous treatment with atropine or hexamethonium. Neither reserpine nor 5-hydroxytryptamine produced gastric haemorrhage in the great majority of vagotomized mice (Table II).

One interpretation of our results with vagotomized mice is that 5-hydroxytryptamine may produce by a peripheral action a mucosal ischaemia which is exacerbated by normal vagal activity. Wilhelmi (1957) found that the ulcerogenic action of 5-hydroxytryptamine in rats could be antagonized by lysergic acid diethylamide, which is known to be a powerful antagonist of the vasoconstrictor action of 5-hydroxytryptamine. Lysergic acid diethylamide has other important actions. Thus mice given this drug after reserpine became hyperactive. We were unable to reduce the incidence of gastric haemorrhage after reserpine in mice by treatment with lysergic acid diethylamide or with its 2-bromo derivative.

The observation that gastric haemorrhage was hardly ever produced by reserpine in vagotomized mice could have been readily explained had it been found that the amount of hydrochloric acid in the stomachs of reserpine-treated mice was decreased by vagotomy. However, no decrease was observed (Table III). The role of gastric acidity in the production of mucosal lesions was made clearer by our experiments with antacids; we found that the repeated oral administration of antacids could reduce substantially the incidence of haemorrhage in reserpine-treated mice. It would thus appear that, whatever be the primary cause of the mucosal lesion, the acid in the stomach helps the development of gross haemorrhage.

That reserpine produces gastric haemorrhage by virtue of a central action is suggested, not only by the results obtained with vagotomized mice, but also by those obtained with reserpine-like drugs;

these had to be given in doses sufficient to produce characteristic central effects for haemorrhage to be obtained regularly with them (Table I).

Haemorrhages, erosions, and acute ulcers of the stomach and duodenum have been obtained by a variety of procedures which cause intracranial damage (Ivy, Grossman, and Bachrach, 1950; Spira, 1955). While it remains to be shown that these acute lesions of the digestive tract result from a specific derangement of nervous function, at least it is clear that they can be produced by mechanisms which affect the central nervous system primarily. Hence it is not unreasonable to suppose that the liability of reserpine-like compounds to cause gastric haemorrhage when they are given in large amounts may be related to their ability to depress the central nervous system in such a way that discharge through the sympathetic division is depressed relative to that through the parasympathetic division. Although no one has shown satisfactorily that *chronic* peptic ulcers can be produced by prolonged stimulation of the vagi or by the prolonged administration of parasympathomimetic drugs, numerous workers have shown that mucosal haemorrhage can be readily produced by simulated vagotonia. Our results with pilocarpine bear out those of several other investigators whose work is discussed by Ivy *et al.* (1950). Why gastric haemorrhage should be obtained under these conditions remains obscure. Ivy *et al.* (1950) think it significant that the acute lesions which are produced in the stomach by interference with the nervous mechanism almost always begin as a focal haemorrhage or ischaemia. It seems that "the vascular, rather than the acid factor, initiates the mucosal injury . . . the primary lesion appears to be the same whatever part of the nervous system is damaged and regardless of whether the disturbance consists of irritation or interruption."

The ability of iproniazid to prevent the production of haemorrhage by either reserpine or 5-hydroxytryptamine provides further support for the view that the ulcerogenic action of reserpine is mediated by 5-hydroxytryptamine. Why iproniazid should act thus is difficult to explain. We originally expected iproniazid to intensify rather than antagonize the ulcerogenic action of 5-hydroxytryptamine, by increasing the amount of free 5-hydroxytryptamine present in the gut through inhibition of mono-amine oxidase. It has since become clear that iproniazid can influence the metabolism of 5-hydroxytryptamine by other means than inhibition of mono-amine oxidase. Thus it can inhibit the decarboxylation of 5-hydroxytryptophan, possibly by virtue of an ability to compete with the co-enzyme pyridoxal-5-phosphate (Westermann, Balzer, and Knell, 1958; Palm, 1958). Moreover, it has been shown that iproniazid may have little effect on peripheral depots of 5-hydroxytryptamine even when given in amounts which produce a two- to three-fold increase in endogenous brain 5-hydroxytryptamine (Udenfriend, Weissbach, and Bogdanski, 1957). We think it significant that previous treatment with iproniazid so changes the response of experimental animals to reserpine that the latter drug causes excitement and hyperactivity instead of sedation (Shore and Brodie, 1957), an observation which was confirmed by us. The fact that some central actions of reserpine can be antagonized by iproniazid makes it less surprising that the ulcerogenic action can also be antagonized by iproniazid, at any rate so long as the latter action is believed to have an important central component.

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THE EXTRACTION OF HUMAN URINARY KININ (SUBSTANCE Z) AND ITS RELATION TO THE PLASMA KININS

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Human urinary kinin (substance Z) has been extracted by modifications of the methods previously described by Gomes (1955) and Jensen (1958). The separation of two oxytocic fractions from such extracts by paper pulp chromatography (Walaszek, 1957; Jensen, 1958) could not be confirmed. Substance Z could not be distinguished from kallidin, bradykinin or glass-activated kinin by parallel quantitative assays, thus confirming that these four substances are very closely related.

Diluted human urine has a marked stimulant effect on the superfused rat uterus (Gaddum, unpublished observations). This finding was followed up by Gomes (1955), who showed that the active principle was a polypeptide similar to bradykinin. More recent chemical evidence suggests that these substances may be different (Gomes, 1957). Werle and Erdös (1954) made similar observations, and called the active principle substance Z. Walaszek (1957) and Jensen (1958) using different chromatographic methods have separated two oxytocic fractions from extracts of human urine. If these fractions really represent different substances, it is important to compare them biologically with each other and with the kinins derived from plasma.

In this investigation, substance Z has been extracted from urine with butanol (Gomes, 1955), by adsorption on IRC-50 (Jensen, 1958) and by various modifications of these methods. Attempts have been made to confirm the presence of two active fractions in such extracts by paper pulp chromatography. Extracts of substance Z have been compared with three kinins prepared from human plasma, kallidin (Werle, 1937; Werle and Berek, 1950), bradykinin (Rocha e Silva, Beraldo and Rosenfeld, 1949) and glass-activated kinin (Armstrong, Jepson, Keele and Stewart, 1954, 1955, 1957). From the results of parallel quantitative assays it is concluded that the active principle in extracts of substance Z is either identical with or closely related to that responsible for the activity of these three plasma kinins.

METHODS

Rat Uterus.—Virgin rats weighing 120 to 200 g. were injected with stilboestrol (10 μg./100 g.) 16 to 18 hr. before use. Uteri were suspended in either a 2 ml. bath or superfusion apparatus (Gaddum, 1953) at 30 to 31°; the de Jalon solution contained atropine sulphate (1 mg./l.).

Guinea-pig Ileum.—A 2 to 3 cm. segment of terminal ileum from animals weighing 120 to 250 g. was either suspended in a 2 ml. bath or superfused using Tyrode solution containing atropine sulphate (1 mg./l.) and mepyramine maleate (1 mg./l.), at 37°.

Rat Stomach.—The method described by Vane (1957) for the estimation of 5-hydroxytryptamine was followed. Male animals of 300 to 400 g. were used; the stomach was suspended in a 10 ml. bath containing oxygenated Tyrode solution at 37°. A dose cycle of 5 min. with 45 sec. contact was satisfactory.

Rat Duodenum.—The proximal 3 cm. of duodenum from rats weighing 150 to 250 g. was suspended in a 2, 10, or 15 ml. bath or superfused, using atropinized (1 mg./l.) de Jalon solution at 30 to 31°. The tissue was stored at 4 to 6° for 2 to 3 hr. before use. A dose cycle of 3 or 5 min. with 30 sec. contact was used.

Rat Ileum.—A 3 cm. segment of terminal ileum from starved rats (150 to 250 g.) was either superfused or suspended in a 2 ml. bath, using atropinized (1 mg./l.) de Jalon solution at 31°. A dose cycle of 5 to 10 min. was used depending upon the speed of response of the preparation, the drug being allowed to act for 40 to 90 sec.

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Rat Colon.—A 3 cm. segment of ascending colon from rats weighing 150 to 250 g. was either superfused or suspended in a 2 ml. bath. Spontaneous activity was very troublesome; it was reduced but by no means abolished by lowering the temperature to 25° and using de Jalon solution. A dose cycle of 3 or 4 min. with 40 to 60 sec. contact was usually satisfactory.

Rat Blood Pressure.—The technique described by Crawford and Outschoorn (1951) was followed, except that the anaesthetic was a mixture of urethane (60 mg./100 g.) and sodium barbitone (50 mg./100 g.) as recommended for the assay of depressor substances by Amin (1953).

Rabbit Blood Pressure.—Animals weighing 1.0 to 2.5 kg. were anaesthetized with sodium barbitone (180 mg./kg.) injected intravenously. The trachea was cannulated and artificial respiration applied when necessary. Blood pressure was recorded from a carotid artery with a mercury manometer and injections were made into a femoral vein. Heparin (30 mg./kg.) was injected intravenously when the dissection was complete.

Adsorption on IRC-50.—Amberlite resin IRC-50 (chromatographic grade and 100 to 200 mesh) was pre-treated by the method of Hirs, Moore, and Stein (1953). Columns 25 to 40 cm. × 2 to 3 cm. were prepared and equilibrated at *p*H 6 with a phosphate buffer. Fresh human urine, filtered and adjusted to *p*H 6, was passed through the column at 10 ml./min. The column was washed with water (about 4 l.) until the washings were clear. The active principle was eluted with either N hydrochloric acid as described by Jensen (1958) or N sodium hydroxide followed by phosphate buffer at *p*H 9. The rate of elution varied in different experiments from 0.5 to 10 ml./min. A 300 cm. head of pressure was necessary in alkaline elutions to achieve a rate of 10 ml./min., the only resistance being that of the column.

Adsorption on Paper Pulp.—Paper pulp columns were prepared by suspending Whatman No. 1 cellulose powder in the solvent (either 7% phenol or water) and allowing the paper to settle by gravity. The dimensions of the column were 12.0×1.7 cm. The extract of substance Z, dissolved in 2 ml. of solvent, was applied to the top of the column, which was then washed with 50 ml. solvent. Elution was attempted with 0.4% v/v acetic acid and then with 0.1N-hydrochloric acid. Washing and elution were maintained at a constant rate (0.5 ml./min.), and 2.5 or 5 ml. samples were collected on a fraction collector.

Butanol Extraction.—The method described by Gomes (1955) for extracting substance Z from urine was followed with minor modifications. Fresh urine was adjusted to *p*H 1.5 with hydrochloric acid, saturated with sodium chloride and extracted with an equal volume of *n*-butanol. The mixture was centrifuged at 2,000 rev./min. for 15 min. and the organic phase transferred to a precipitation jar, from which the air had been displaced with nitrogen. Five vol. cooled diethyl ether (peroxide-free) were added and the precipitate allowed to settle for 3 to 4 hr. at 4 to 6°. The supernatant was then removed by suction and the precipitate dried *in vacuo*.

Standard Substance Z.—An extract of substance Z prepared by the butanol method above was used as the master standard, 1 mg. being called 1 unit. Substandards were prepared by butanol extraction, IRC-50 adsorption or by a combination of these methods and were standardized against the master standard by 2 + 2 dose assay on the rat uterus.

Preparation of Kallidin. — Freeze-dried human plasma (citrated) was re-constituted with water, and incubated for 10 min. at 37° with N hydrochloric acid (1 in 10) to destroy the kininase and kallikrein inactivator (Horton, 1958). The plasma was then adjusted to *p*H 7.3 with sodium bicarbonate, and incubated at 37° for 2 hr. with human urinary kallikrein (30 mu./ml.). The kallidin was extracted by pouring the plasma into 2 vol. boiling absolute alcohol and boiling for 5 min. The mixture was centrifuged and the supernatant decanted off. The residue was re-extracted with 66% alcohol and the combined supernatants were evaporated to dryness *in vacuo*.

Preparation of Glass-activated Kinin.—Polythene containers and pipettes were used throughout the initial stages. Freshly collected heparinized (10 units/ml.) human blood was centrifuged at 3,000 rev./min. for 1 hr. at 4°. The plasma was transferred to another tube and re-centrifuged. The upper ¼ was pipetted into an unsiliconed glass tube and stirred with a glass rod. The development of activity was followed on the rat uterus. After 20 min. contact, the kinin was extracted with alcohol as described for kallidin. The final residue was shaken with ether to remove lipids, and re-dried *in vacuo*.

Bradykinin.—This was prepared by the action of trypsin on ox globulins. It had an activity of 7.1 units/mg. and was kindly supplied by Dr. M. Schachter.

Preparation of Human Urinary Kallikrein.—Pooled male human urine (5 l.) was concentrated to a fifth of its volume in a climbing film evaporator. The concentrate was dialysed through cellophane against running tap water for 48 hr. The kallikrein was then precipitated by the addition of 4 vol. acetone at 4°. The precipitate was separated and dried *in vacuo*. The amorphous material was powdered and standardized against a sample of Padutin (Bayer) kindly supplied by Dr. E. Werle.

Results

Butanol Extraction of Substance Z from Human Urine

Substance Z was extracted from urine with butanol by the method of Gomes (1955). The recovery was estimated by comparing the activity of the extract with that of the crude urine. In different experiments yields varied from 0.5 to 10% in contrast to the 100% reported by Gomes. The reasons for this discrepancy were investigated.

It was possible that substance Z is unstable at the *p*H (1.5) of extraction. However, since urine adjusted to *p*H 1 could be boiled for 1 hr. without detectable loss of activity, that possibility was excluded. Extraction with 1 vol. *n*-butanol might be incomplete. When the separated butanol layer (after extraction) was shaken with water, 80 to 100% of the original substance Z was found in the aqueous phase. This suggested that the loss of activity must occur during the ether precipitation.

If the ether was allowed to stand exposed to the air either before or during the precipitation, recoveries were always poor (0.5 to 2%). If fresh ether was used and the precipitation carried out in an atmosphere of nitrogen, recoveries of 10% were invariably obtained. It seemed probable that ether precipitation was an inefficient method of obtaining substance Z from the butanol phase.

It was found that recoveries of 80 to 90% could be obtained by evaporating the butanol phase to dryness *in vacuo* at 50° using a capillary air inlet. Although more of the active material was recovered than with ether precipitation, there was a corresponding increase in the amount of impurities, and so the final dried powder was only slightly more active on a weight basis than that obtained by ether precipitation.

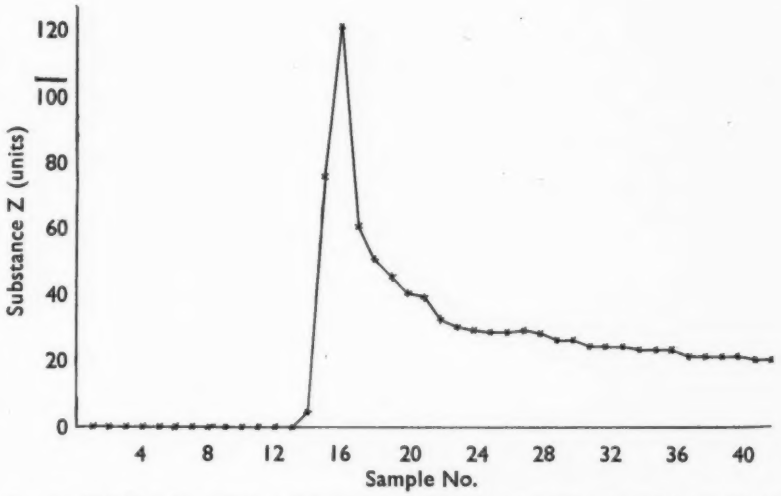

FIG. 1.—IRC-50 column 37·0 × 2·5 cm. 3 l. of human urine (10 units substance Z/ml.) applied at *p*H 6. Elution (1 ml./min.) with N hydrochloric acid. Recovery (samples 14 to 42)=32%. Each sample was 25 ml. in volume.

Adsorption of Substance Z on IRC-50

Jensen (1958) has shown that the Amberlite resin IRC-50 is suitable for extracting substance Z from large volumes of urine. He eluted the active principle with hydrochloric acid and obtained recoveries up to 60%.

Several experiments were performed using this method. Recoveries varied from 10 to 50%. These low figures were partly the result of considerable tailing, which persisted even when the elution rate was slow (0.5 ml./min.) (Fig. 1). In this way large volumes of eluate containing a relatively low concentration of substance Z were obtained. Since one of the purposes of using the column was to concentrate the activity into smaller volumes, it was important to find a way of overcoming this difficulty.

Elution of substance Z, a polypeptide, from IRC-50 with acid depends upon depressing the ionization of the column. If the *p*H is raised the capacity of the column for cations is increased, but the peptide is converted into an anionic form and will not be retained. This proved to be a good way of eluting substance Z from IRC-50.

Batchwise experiments showed that substance Z could be eluted from IRC-50 at *p*H 9. Sodium hydroxide was added in an amount calculated from previous titrations to raise the *p*H of the column from 6 to 9. In a batchwise experiment, the resin would rapidly equilibrate at this *p*H, but when carried out on a column a *p*H gradient was established, the upper parts being more alkaline. M sodium phosphate buffer (*p*H 9) was added after the sodium hydroxide. As the buffer passed through the *p*H was readjusted to 9, alkali being displaced to raise the *p*H lower down. Ultimately, the whole column reached *p*H 9, and at that point a small number of highly active fractions were collected with recoveries of 80 to 100% (Fig. 2). Since substance Z is rather unstable in alkali, elution was carried out rapidly (10 ml./min.), some protection from the alkaline solvent front being provided by the buffering action of the column.

Elution could also be effected with phosphate buffer alone, but large quantities of buffer were required, elution was much slower and the peak was not so sharp.

Eluate samples from IRC-50 columns were extracted by the butanol method described above, and the butanol phase was evaporated to dryness. Good recoveries were obtained. The final dried powder was stable and contained 10 to 15 units/mg.

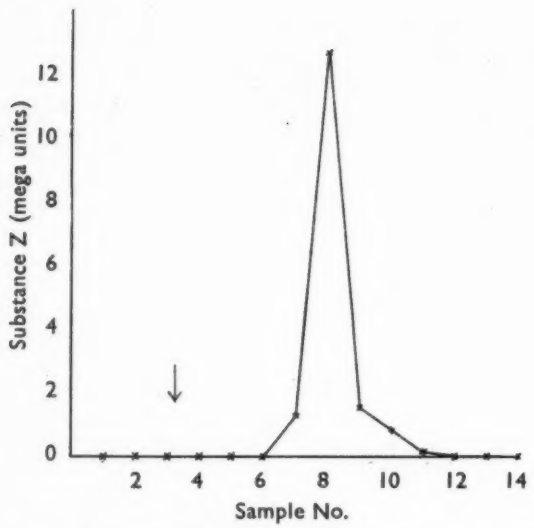

FIG. 2.—IRC-50 column 27·5 × 2·0 cm. 2 l. of human urine (10 units substance Z/ml.) applied at *p*H 6. Elution (10 ml./min.) with 80 milliequivalents sodium hydroxide followed by sodium phosphate buffer (*p*H 9) at arrow. Recovery=82%. Sample volume, 25 ml.

Paper Pulp Chromatography

Numerous experiments were performed in an attempt to repeat the work of Walaszek (1957) and Jensen (1958) in which two fractions were separated from substance Z extracts by paper pulp chromatography.

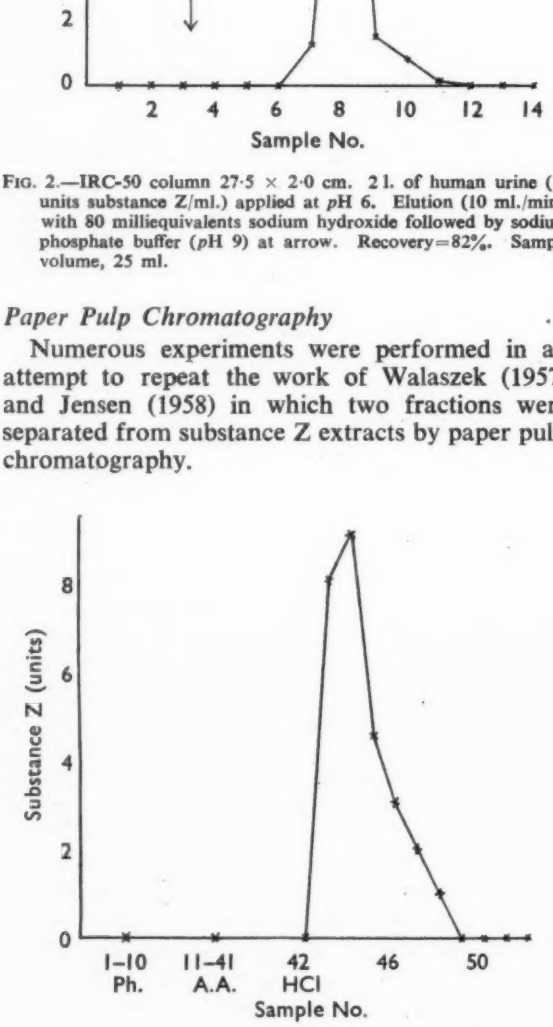

FIG. 3.—Paper pulp column 12·5 × 1·7 cm. prepared by suspending cellulose powder in 7% phenol. 55 units of substance Z extract, dissolved in 2 ml. 7% phenol, applied. Column washed with 50 ml. 7% phenol (**Ph.**). Elution with 150 ml. 0·4% v/v acetic acid (**A.A.**) followed by 50 ml. 0·1 N hydrochloric acid (**HCl**) at 0·5 ml./min. Recovery=50%. Sample volume, 5 ml.

A typical result obtained when using 7% phenol as the solvent is shown in Fig. 3. An active fraction corresponding to Walaszek's Z_1 could not be eluted with 0.4% v/v acetic acid, using up to 250 ml., whereas a single peak of activity appeared on elution with 0.1 N hydrochloric acid, corresponding to Walaszek's Z_2 fraction.

When water was used instead of 7% phenol, results similar to those reported by Jensen (1958) were obtained. A peak of activity could be washed through the column with water, and a second peak occurred on elution with 0.1 N hydrochloric acid (Fig. 4). It seemed probable that the first peak represented excess substance Z from overloading the column ; this was confirmed in two ways.

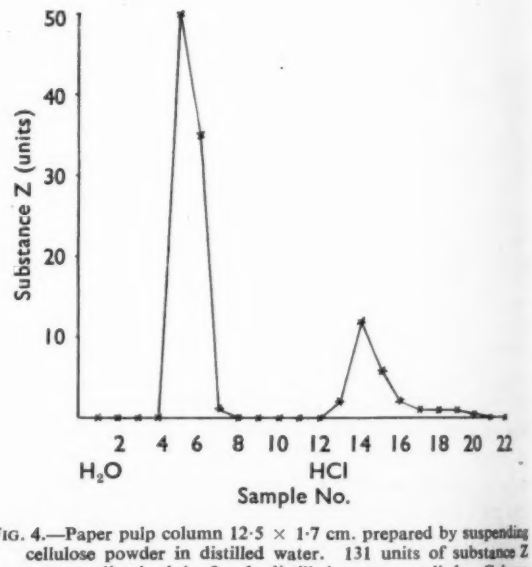

FIG. 4.—Paper pulp column 12·5 × 1·7 cm. prepared by suspending cellulose powder in distilled water. 131 units of substance Z extract, dissolved in 2 ml. distilled water, applied. Column washed with distilled water (H_2O). Elution with 0·1 N hydrochloric acid (**HCl**) at 0·5 ml./min. Total recovery (washings plus eluates)=87%. Sample volume, 5 ml.

The experiment illustrated in Fig. 4 was repeated. Columns of the same size were used, but the amount of substance Z extract applied was reduced from 131 units to 88 and 38 units respectively in the two experiments. The results are shown in Fig. 5 (cf. Fig. 4). Approximately the same amount of activity, about 30 to 35 units, was eluted with hydrochloric acid from each of the three columns. On the other hand the size of the first peak fell from 86 to 42 to 0 units. Clearly, the capacity of a paper pulp column of these dimensions was about 35 units, and if more than this was applied the excess passed through in the aqueous washings.

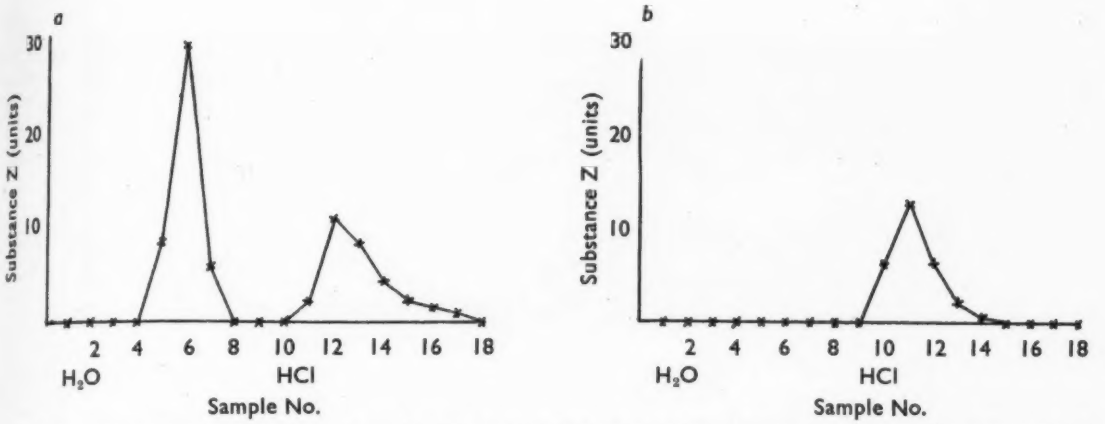

FIG. 5.—Columns prepared as in Fig. 4. In (*a*) 88 units and (*b*) 38 units of substance Z extract applied. Columns washed with distilled water (H_2O). Elution with 0·1 N hydrochloric acid (HCl) at 0·5 ml./min. Total recoveries (*a*) 90%, (*b*) 72%.

Active material (30 units) which passed unadsorbed through one column was applied to a second. All the substance Z was adsorbed, and most of it could be recovered by hydrochloric acid elution.

Thus the separation of two active fractions on paper pulp using 7% phenol could not be confirmed, and the appearance of two peaks when phenol was omitted was shown to be due to overloading of the column.

Substance Z and Kallidin

Kallidin is the plasma kinin formed by the action of kallikrein on plasma (Werle, 1937). Since it is said to differ from substance Z (Werle and Erdös, 1954), parallel quantitative assays were performed (Fig. 6). The results on four tissues are shown in Table I. Each figure represents the mean of three assays with the standard error. From these parallel assays it is clear that kallidin and substance Z must be closely related.

TABLE I

UNITS OF SUBSTANCE Z EQUIACTIVE WITH 1 MG. KALLIDIN

	Units (±s.e.)
Guinea-pig ileum	12·0±3·5
Rat duodenum	9·2±1·2
,, ileum	8·7±0·3
,, uterus	8·1±0·6

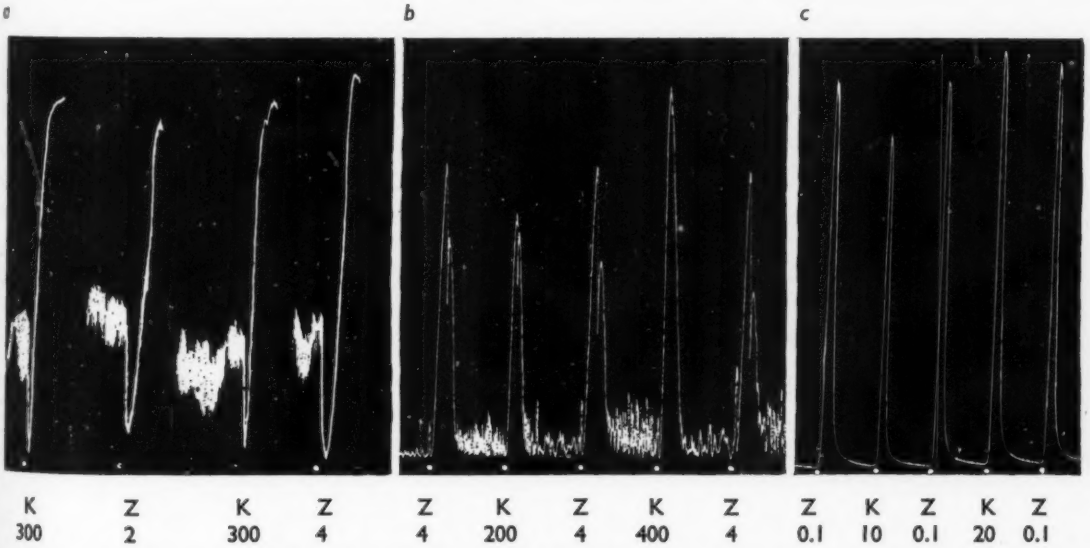

FIG. 6.—(*a*) Rat ileum suspended in a 2 ml. bath containing atropinized (10^{-6}) de Jalon solution at 31°. (*b*) Guinea-pig ileum suspended in a 2 ml. bath containing atropine (10^{-6}) and mepyramine (10^{-6}) in Tyrode solution at 37°. (*c*) Rat uterus suspended in a 2 ml. bath containing atropinized (10^{-6}) de Jalon solution at 30°. K=human kallidin extract (μg.). Z=substance Z extract (units).

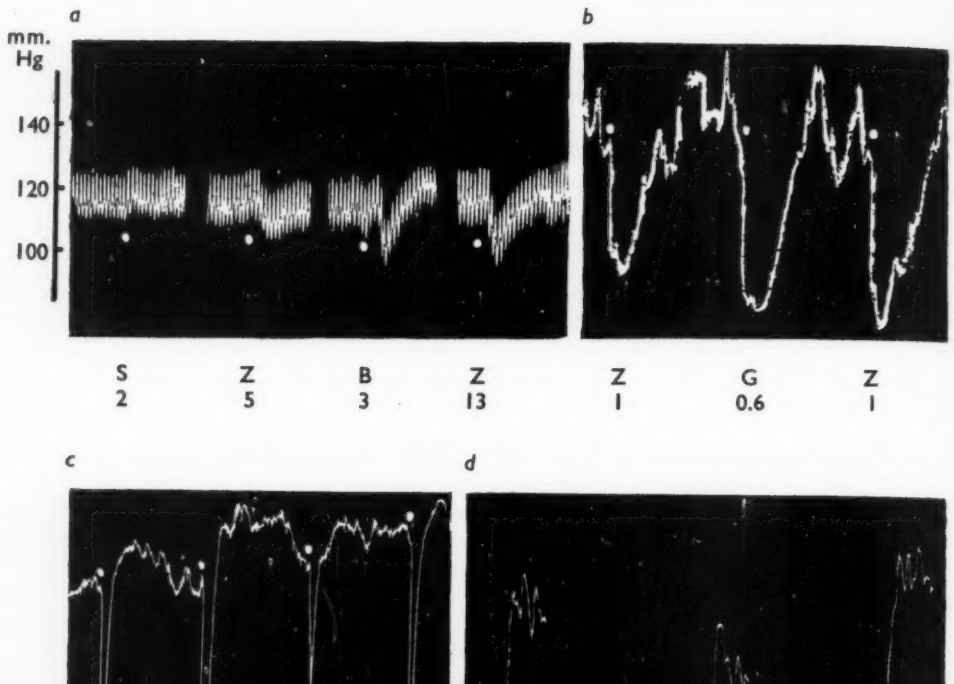

FIG. 7.—(*a*) Rabbit 1·5 kg. anaesthetized with intravenous sodium barbitone (180 mg./kg.). Blood pressure recorded from carotid artery with a mercury manometer. Heparin (45 mg.) was injected intravenously as anticoagulant. (*b*) Rat colon suspended in a 2 ml. bath containing de Jalon solution at 25°. (*c*) Rat duodenum suspended in a 2 ml. bath containing atropinized (10^{-6}) de Jalon solution at 31°. (*d*) Rat stomach suspended in 10 ml. bath containing oxygenated Tyrode solution at 37°. S=saline (ml.). Z=substance Z extract (units). B=bradykinin (units). G=glass-activated kinin (mg.).

Substance Z and Bradykinin

Bradykinin is the plasma kinin formed by the action of trypsin on plasma (Rocha e Silva *et al.*, 1949). It has been shown that substance Z is a similar polypeptide (Gomes, 1955, 1957). The two substances have been compared by parallel assays on six different biological preparations. Both stimulate the rat uterus, guinea-pig ileum, rat ileum and rat stomach, and both cause relaxation of the rat duodenum and a fall of the rabbit blood pressure (Fig. 7).

The values in Table II differ among themselves, but are not inconsistent with the view that the same active principle is responsible for the activity of the two extracts. The index of discrimination (Gaddum, 1955) is never higher than 2.5.

TABLE II

UNITS OF SUBSTANCE Z EQUIACTIVE WITH 1 UNIT BRADYKININ

	Units (±s.e.)
Guinea-pig ileum	10·0±3·0
Rat ileum	7·0±0·8
,, uterus	6·2±0·5
,, stomach	4·5±1·2
,, duodenum	4·4±1·0
Rabbit blood pressure	4·0±1·1

Substance Z and Glass-activated Kinin

When human plasma is exposed to a glass surface, a kinin is formed which causes pain and stimulates the rat uterus (Armstrong *et al.*, 1954, 1955, 1957). This substance is very similar to bradykinin (Jepson, Armstrong, Keele and Stewart, 1956). In view of the similarity of substance Z, it was of interest to compare it also with glass-activated kinin. The relative potencies obtained by parallel assays on six tissues are shown in Table III. In this series only one assay was performed on each tissue and the error represents the limits of a bracketing assay. In general the values agree well enough ; 0.6 would fall within the error range of them all. It would seem that substance Z and the glass-activated kinin are also closely related.

TABLE III

UNITS OF SUBSTANCE Z EQUIACTIVE WITH 1 MG. GLASS-ACTIVATED KININ

	Units
Guinea-pig ileum	1·2±0·6
Rat duodenum	1·0±0·5
„ colon	1·0±0·4
„ ileum	0·8±0·3
„ uterus	0·5±0·2
„ blood pressure	>0·3

DISCUSSION

Extraction of Substance Z

Extraction of substance Z from human urine using the method described by Gomes (1955) gave poor yields. Precipitation with ether appears to be an inefficient way of recovering the active material from the butanol phase ; much better recoveries have been obtained by evaporating the butanol to dryness *in vacuo*, and the latter procedure has been adopted in the final method.

Elution of substance Z from IRC-50 with alkali was found to be more satisfactory than the acid elution used by Jensen (1958). Recoveries were good and the active material was concentrated in very few fractions. As pointed out by Jensen (1958), columns of IRC-50 have the advantage that substance Z can be adsorbed very conveniently from large volumes of urine. If elution is carried out with alkali, little activity is lost and the dried samples contain inorganic phosphate as the main impurity. If the eluate samples are then extracted with butanol, little inorganic material passes into the organic phase and thus the main impurity is left behind. The extracts prepared by this combined method are less active but more stable than those prepared by Jensen (1958) using IRC-50 and paper pulp columns.

Paper Pulp Chromatography

Walaszek (1957) applied substance Z extracts to paper pulp columns in 7% phenol and was able to elute two peaks of activity with 0.4% acetic acid. He subsequently found that the second peak could be eluted more readily with 0.1 N hydrochloric acid, with a total recovery of 60 to 90%. In the present investigation no activity could ever be eluted with acetic acid and this accounted for the failure to separate two fractions. The active material could be eluted readily by 0.1 N hydrochloric acid in a single peak with yields of 50 to 70%. The difference of these findings from those of Walaszek (1957) may have been due to slight differences in technique or materials ; it is interesting, however, that Jensen (1958) was also unable to recover a Z_1 fraction by this method.

Similar results were obtained if this type of experiment was performed using water instead of 7% phenol as the solvent. An apparent separation of two fractions (see Jensen, 1958) was shown to be due to overloading of the column. It provided no evidence for the presence of two different substances. These negative results do not, however, exclude such a possibility. Jensen (1958) produced some evidence to show that two fractions could also be separated by chromatography on an alumina column. That type of separation was not attempted in the present investigation.

Substance Z and the Plasma Kinins

Werle and Erdös (1954) concluded that substance Z and kallidin were different substances. Their evidence was that substance Z dialysed more slowly and that it was rather more stable to alkali than kallidin. Both these differences might be accounted for by differences in purity of the two extracts. In addition, they found that substance Z had no effect on the rat intestine unlike kallidin and bradykinin which stimulate that preparation. In the present investigation extracts of substance Z caused relaxation of the rat duodenum and rat colon, and contraction of the rat ileum, similar effects being observed with kallidin and bradykinin. It is possible that Werle and Erdös (1954) used too little substance Z in their experiments with the intestine.

Substance Z and kallidin could not be distinguished by parallel assays on four tissues suggesting that they may contain the same active principle. This is supported by the similar results with bradykinin, which is possibly identical with kallidin (Werle, 1953 ; Holdstock, Mathias, and Schachter, 1957 ; Mathias and Schachter, 1958).

The relative potencies of substance Z to bradykinin on the rabbit blood pressure, rat uterus and guinea-pig ileum (Table II) show certain similarities to those obtained by Gomes (1957). In both investigations substance Z appeared to be relatively more active than bradykinin on the blood pressure and relatively less active on the ileum, the uterus being intermediate. These differences are small, but they may be significant if, as Gomes (1957) suggests from counter-current distribution experiments, the active principles are different.

The kinin prepared by glass-activation of human plasma was not tested for its pain-producing activity and so is referred to here as glass-activated kinin and not as pain-producing substance (Armstrong *et al.*, 1954). This kinin is known to be very similar to bradykinin (Jepson *et al.*, 1956; Armstrong *et al.*, 1957), and its similarity to substance Z on parallel assays provided more evidence of the close relationship of all these peptides.

From these biological comparisons it is concluded that the same active principle may be responsible for the activity of the four kinins studied, but the question must be settled ultimately by chemical analysis of the isolated peptides.

In the meantime there seems little justification for the continued use of the term "substance Z," and it is suggested that it should be replaced by "urinary kinin," analogous to the terms plasma kinin (Lewis, 1958) and wasp kinin (Schachter and Thain, 1954), thus emphasizing their similarity without implying identity.

We are grateful to Dr. R. A. Cumming, Regional Director of the Blood Transfusion Service, the Royal Infirmary, Edinburgh, for supplies of blood and plasma.

The work reported in this paper was done during the tenure by one of us (E. W. H.) of a Graduate Research Scholarship, Edinburgh University, and forms part of a Thesis submitted for the degree of Ph.D.

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HUMAN URINARY KININ EXCRETION

BY

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(RECEIVED NOVEMBER 18, 1958)

It has been shown that the isolated rat duodenum relaxes in the presence of low concentrations of plasma and urinary kinin. The tissue is at least as sensitive as the rat uterus. Vasopressin and oxytocin, in large doses, also caused relaxation of the duodenum whereas acetylcholine, substance P and 5-hydroxytryptamine caused contraction. It was concluded that if an extract is assayed on the rat uterus and the rat duodenum in parallel using plasma kinin as a standard, and the results agree, this is good evidence that the active principle being estimated is a kinin. This method is therefore both sensitive and specific for kinin estimations, but it will not distinguish between kinins of different origin. The urinary excretion of kinin in 14 healthy adults was found to be fairly constant. The minute output was unaffected by the rate of urine formation, urinary *p*H, or time of day. There was no increase during sweating or salivation.

The plasma kinins are polypeptides derived from plasma which stimulate certain types of plain muscle. They include the substances kallidin (Werle, 1937; Werle and Berek, 1950), bradykinin (Rocha e Silva, Beraldo and Rosenfeld, 1949), and the pain-producing substance (Armstrong, Jepson, Keele, and Stewart, 1954, 1957). There is some evidence that the same active principle may be responsible for the activity of all these substances (Werle, 1953; Jepson, Armstrong, Keele, and Stewart, 1956; Holdstock, Mathias and Schachter, 1957), and possibly also for the activity of various other bradykinin-like substances which have been reported (Hilton and Lewis, 1955, 1956; Schachter, 1956; Adam, Hardwick, and Spencer, 1957; Lewis and Work, 1957; Lewis, 1958). Human urine contains a similar kinin which could not be distinguished from the plasma kinins qualitatively or by parallel assays (Gomes, 1955; Gaddum and Horton, 1959) though there is some chemical evidence that the active principles are different (Gomes, 1957).

There is evidence for the formation of a plasma kinin during functional vasodilatation in the salivary and sweat glands (Hilton and Lewis, 1955, 1956; Fox and Hilton, 1958), and it is possible that kinin formation may occur in other parts of the body. The excretion of a kinin in normal human urine suggested a continual production, but it was not known to what extent the excretion rate varied under different physiological conditions and in different people. This has been investigated using a new method for kinin estimations which is both sensitive and specific. The amount of kinin excreted/unit time was fairly constant in both male and female urine.

METHODS

Rat Uterus.—Virgin rats weighing 120 to 200 g. were injected with stilboestrol (10 μg./100 g.) 16 to 18 hr. before use. Uteri were suspended in a 2 ml. bath at 30 to 31° and bathed in de Jalon solution containing atropine sulphate (10^{-6}).

Rat Duodenum.—The proximal 3 cm. of duodenum from rats weighing 150 to 250 g. were suspended in a 10 or 15 ml. bath containing atropinized (10^{-6}) de Jalon solution. It was observed that tissues kept at 4 to 6° for 2 to 3 hr. before use behaved particularly well, so this procedure has been used routinely. Since relaxation of the muscle was small, a high lever magnification (1 in 15) was required. A tension of approximately 0.5 g. was suitable for most tissues. A bath temperature of 30 to 31° was used to minimize spontaneous activity, but temperatures up to 37° were also satisfactory. De Jalon solution was the most satisfactory; increasing the calcium concentration did not improve performance nor did the use of Tyrode solution. A dose cycle of 3 or 4 min. was used with 30 sec. contact time. The bath was washed out by upward displacement and overflow.

Simultaneous Parallel Assays.—The rat uterus was suspended in a 2 ml. bath and the rat duodenum in a 10 or 15 ml. bath. The two tissues were used

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simultaneously for the assay of urine samples. In each case a dose cycle of 4 min. was used, doses being administered to each tissue alternately every 2 min.

Collection of Urine Specimens.—Urine was collected over a timed period as follows. The subject emptied his bladder and noted the time, the specimen being discarded. At the next micturition, the whole specimen was collected and the time again noted. The volume and *p*H (B.D.H. narrow range indicator paper) of the urine were measured immediately. A 10 ml. aliquot was transferred to a boiling tube and frozen by immersion in a mixture of acetone and solid carbon dioxide ("Drikold") at −70° within 30 min. of collection. It was then stored in the deep freeze until the time of the assay.

Alumina Columns. — All urine specimens were passed through an alumina column before assay. Columns 6.5 × 1.0 cm. were prepared by suspending 5 g. alumina (Savory and Moore) in water and allowing it to settle by gravity. 5 ml. urine was added and allowed to run through at 1 ml./min. When all the urine had passed into the column, distilled water was added. Washing was carried out at 1 ml./min. and 5 ml. fractions were collected; the first was discarded, while the second and third fractions which contained all the activity were pooled and used for biological assays. If the interval before the assay was to be longer than 30 min. the filtrates were frozen and stored in the deep freeze.

Standard Urinary Kinin.—This was prepared from human urine by IRC-50 adsorption and butanol extraction (Gaddum and Horton, 1959). 1 mg. of the original master standard was called 1 unit.

Standard Plasma Kinin.—This was prepared by the action of human urinary kallikrein on acid-treated human plasma, the kinin (kallidin) being extracted with alcohol (Gaddum and Horton, 1959).

Substance P.—This was prepared and kindly supplied by Dr. T. B. B. Crawford.

Wasp Kinin and Ox Bradykinin.—These materials were prepared and kindly supplied by Dr. M. Schachter.

Vasopressin, Oxytocin, and Angiotensin. — The following commercial preparations of these substances were used: Pitressin (Parke, Davis), Pitocin (Parke, Davis), and Angiotonin (Eli Lilly).

Results

The Rat Duodenum

If a tissue extract is assayed on a single preparation, for example the rat uterus, against standard plasma kinin, there can be no certainty that the active principle being estimated is really plasma kinin; some other test of identification is necessary. One of the best ways to ensure specificity is to assay an unknown in parallel on several different tissues. If similar results are obtained in this way it is reasonably certain that the active principle in the extract and the standard preparation are identical (Chang and Gaddum, 1933).

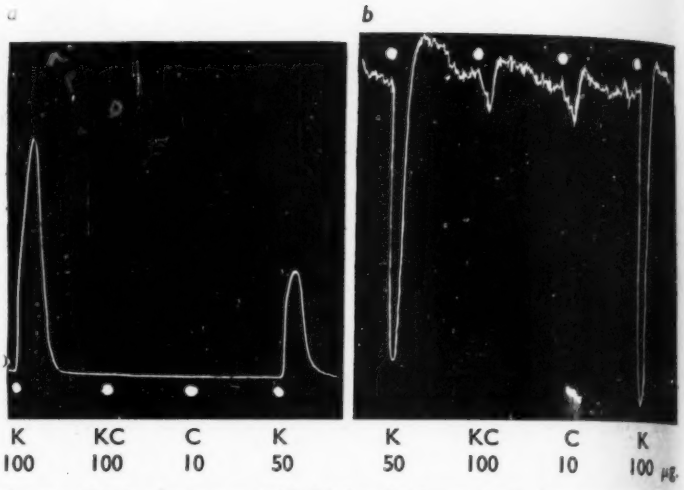

FIG. 1.—(*a*) Isolated rat uterus; (*b*) isolated rat duodenum. Each tissue was suspended in a 10 ml. bath containing atropinized (10^{-6}) de Jalon solution at 30°. *K*=standard plasma kinin extract. *KC*=mixture of plasma kinin (100 µg.) and chymotrypsin (10 µg.) incubated for 1 hr. at 37° and then heated in a boiling water bath for 10 min. *C*=control containing no plasma kinin treated as *KC*. All doses in µg.

It has been found that the rat duodenum responds to small doses of plasma kinin, being at least as sensitive as the rat uterus, and usually more sensitive. Unlike most plain muscle, the rat duodenum relaxed in the presence of kinin (Fig. 1). That this inhibitory action and the stimulation of other types of plain muscle were produced by the same active principle has been confirmed by parallel assays using kinin preparations of different potency and of different origin. The inhibitory action on the duodenum like the stimulant action on the uterus was abolished by incubation with chymotrypsin (Fig. 1).

The Action of Other Oxytocic Polypeptides on the Rat Duodenum

Vasopressin and oxytocin had no action on the rat duodenum in concentrations comparable to those which stimulate the rat uterus. In very high concentrations (100 mU./ml.) both these polypeptides had a qualitatively similar effect to plasma kinin (Fig. 2). Parallel assays of vasopressin and oxytocin with plasma kinin were performed on the rat uterus and rat duodenum;

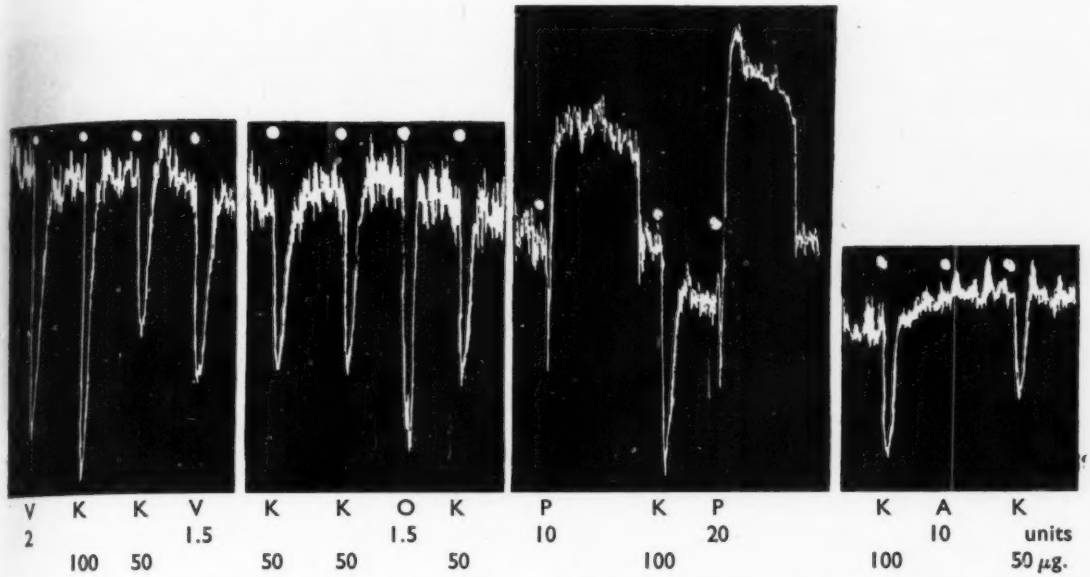

FIG. 2.—Isolated rat duodenum suspended in a 15 ml. bath containing atropinized (10^{-6}) de Jalon solution at 30°. *V*=vasopressin (units). *O*=oxytocin (units). *P*=substance P (units). *A*=angiotensin (units). *K*=standard plasma kinin extract (μg.).

the results are shown in Table I. Clearly there is a great discrepancy between the results on the two tissues, and the index of discrimination (Gaddum, 1955) is very high. Parallel assays using these two tissues will certainly distinguish vasopressin and oxytocin from plasma kinin in a tissue extract.

TABLE I

UNITS OF OXYTOCIC POLYPEPTIDES EQUIACTIVE WITH 1 MG. STANDARD PLASMA KININ

	Rat Uterus	Rat Duodenum	Index of Discrimination
Vasopressin	0·15	15	100
Oxytocin	0·015	22·5	1,500
Angiotensin	12·5	>200	>16
Substance P	243	Contracts	—

Substance P stimulated the rat duodenum, although some extracts had a very transient initial inhibitory effect (Fig. 2). The stimulant action was not due to contamination with histamine because the effect was not inhibited by mepyramine maleate (10^{-6}).

Angiotensin [hypertensin, angiotonin] (Braun-Menéndez and Page, 1958) had no action on the duodenum in the largest doses used (Fig. 2), whereas it was active on the rat uterus. The index of discrimination is over 16 (Table I), showing that these two tissues will easily distinguish between angiotensin and plasma kinin.

Other kinins have been compared with the standard plasma kinin on these two preparations (Fig. 3). Neither bradykinin, the plasma kinin formed by the action of trypsin on plasma, nor the human urinary kinin (substance Z) nor wasp kinin could be distinguished from the standard plasma kinin (kallidin), the index of discrimination in each case not differing significantly from 1.0 (Table II).

TABLE II

AMOUNTS OF KININS EQUIACTIVE WITH 1 MG. STANDARD PLASMA KININ

Amounts are given with standard errors.

	Rat Uterus	Rat Duodenum	Index of Discrimination
Bradykinin (units) ..	1·3±0·3	1·5±0·5	1·2
Urinary kinin (units) ..	8·1±0·5	9·2±1·2	1·1
Wasp kinin (μg.) ..	1·2±0·4	1·2±0·4	1·0

The Action of Non-peptide Substances on the Rat Duodenum

Acetylcholine and 5 - hydroxytryptamine stimulate the rat uterus and both were found to stimulate the rat duodenum (Fig. 4). Histamine in large doses also stimulated the rat duodenum. Adrenaline and noradrenaline inhibited the duodenum, concentrations of 10^{-6} being required. Adenosine and its monophosphate both inhibited the rat duodenum, but the maximum relaxation which they could produce was less than half the maximum which could be reached with plasma kinin (Fig. 4). Thus none of these substances behaved like plasma kinin on both the rat uterus and the rat duodenum.

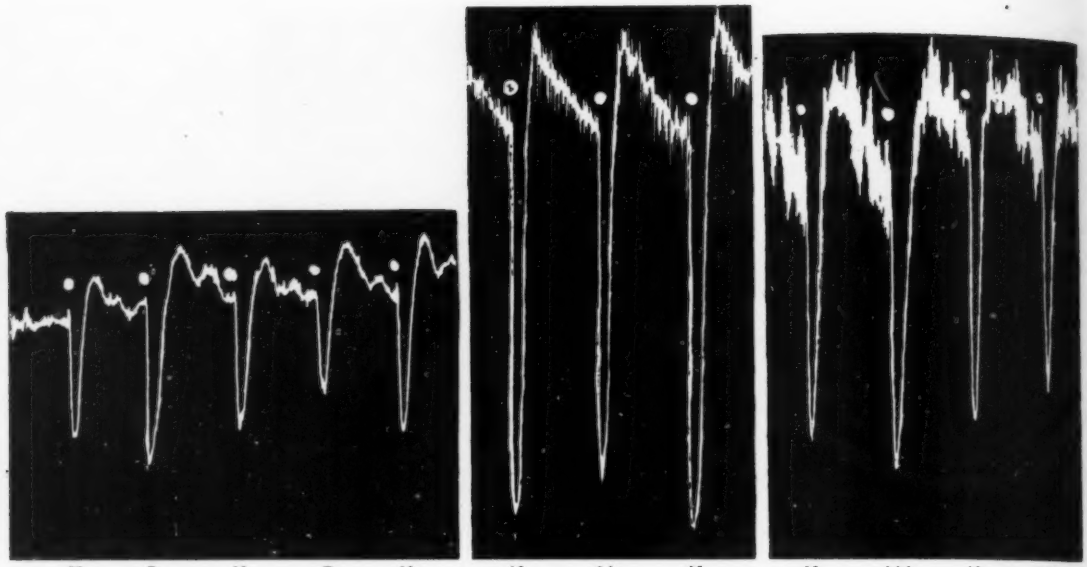

FIG. 3.—Isolated rat duodenum suspended in a 15 ml. bath containing atropinized (10^{-6}) de Jalon solution at 30°. ***K***=standard plasma kinin extract (μg.). ***B***=ox bradykinin (units). ***U***=urinary kinin (units). ***W***=wasp kinin (μg.).

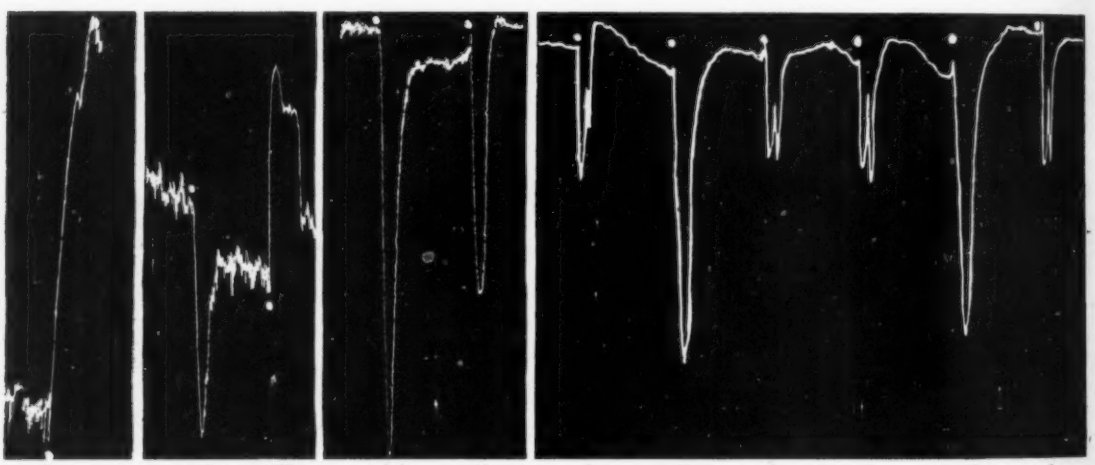

FIG. 4.—Isolated rat duodenum suspended in a 10 ml. bath containing de Jalon solution at 30°. ***Ac***=acetylcholine. ***K***=standard plasma kinin extract. ***HT***=5-hydroxytryptamine creatinine sulphate. ***Ad***=adrenaline hydrochloride. ***A***=Adenosine. Doses in μg.

The Estimation of Urinary Kinin

The kinin in human urine was originally detected by testing diluted crude human urine on the superfused rat uterus. When parallel assays were performed on the rat uterus and the rat duodenum using crude urine, the results were often markedly different, the apparent potency of the urine being up to five times greater on the duodenum. The cause for this discrepancy was found to be a substance in urine which inhibits the duodenum but does not stimulate the uterus. This inhibitory substance has not been identified, but it can be removed from urine by adsorption on alumina.

A sample of crude urine was assayed on the uterus and duodenum before and after passage

through an alumina column. The results of the assay with the rat uterus, expressed in units/ml. of kinin, before and after alumina treatment were 6 (±2 s.e.) and 15 (±5) and with the rat duodenum 30 (±6) and 15 (±5). After the alumina treatment, not only was the duodenum estimate lower but the uterus value was appreciably higher, suggesting that the inhibitory substance may also depress the stimulant action of kinin on the uterus. This result illustrated very clearly the unreliability of a single tissue for these estimations.

The recovery of urinary kinin from alumina columns was investigated by applying a standard extract dissolved in 5 ml. water and washing the column with water. 2.5 ml. samples were collected on a fractionator. Urinary kinin passed through the column and appeared in the filtrate as soon as the hold-up volume had been displaced (Fig. 5). Recoveries were always over 90%.

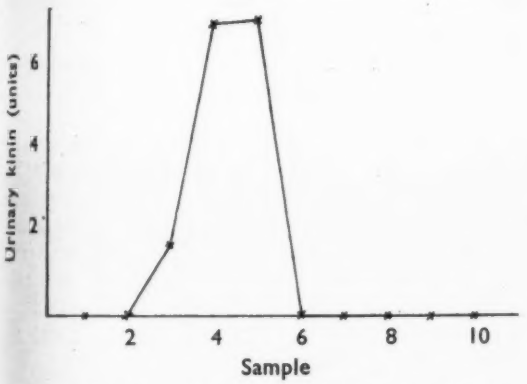

FIG. 5.—Alumina column 6·5×1·0 cm. 16 units urinary kinin were applied in 5 ml. distilled water. The column was washed with distilled water. Recovery=95%. Sample volume, 2·5 ml.

Stability of Urine Samples

Jensen (1958) showed that extracts of urinary kinin in solution were more stable than crude urine. This observation has been confirmed. Since a kinin-inactivating enzyme has been detected in urine (Frey, Kraut, and Werle, 1950), it was thought probable that this might account for the relative instability of crude urine.

The urinary kinin content of two urine specimens was estimated by the method described above. Each specimen was then divided into three portions. One portion was adjusted to *p*H 7, the second to *p*H 2 and the third was heated at *p*H 6 in a boiling water bath for 10 min. and adjusted to *p*H 7. The specimens were left for 24 hr. at room temperature, and their kinin content was then estimated (Table III). The loss of activity in the acidified and pre-heated specimens was far less than in the untreated specimens.

TABLE III

RECOVERY OF KININ FROM URINE SAMPLES LEFT AT 10 TO 15° FOR 24 HR.

The recovery is given as %.

Treatment	Expt. 1	Expt. 2
*p*H 2	100	77
Boiled 10 min. then *p*H 7	80	72
*p*H 7	3	18

These results strongly suggest that in crude urine kinin was destroyed by an enzyme, which could be inactivated by heat and was inactive at a low *p*H.

Urine specimens which were frozen and stored in a deep-freeze showed no detectable loss of activity over a two-month period. The urinary kininase was evidently inactive at this temperature. All specimens were therefore frozen within 30 min. of collection unless they were to be assayed immediately.

Individual Variation in Urinary Kinin Excretion

The urinary excretion of kinin was estimated in 14 healthy adults, 12 male and 2 female. All except one (subject 9) were within the age range 16 to 40 years. The results are shown in Table IV.

TABLE IV

THE URINARY EXCRETION OF KININ IN 14 HEALTHY ADULTS, ESTIMATED ON THE RAT UTERUS AND RAT DUODENUM

The kinin values are expressed in units/min. with s.e. and the number of specimens tested is given in parentheses. An asterisk indicates a female subject.

Subject No.	Rat Uterus	Rat Duodenum
1	12·8± 5·9 (46)	8·2±4·8 (40)
2*	9·0± 5·5 (30)	5·7±6·1 (20)
3	12·3± 8·3 (6)	13·8±7·8 (4)
4	15·8± 2·1 (5)	11·2±3·3 (5)
5	6·5± 2·9 (8)	4·9±2·3 (8)
6	15·1± 6·0 (5)	11·0±4·5 (5)
7	12·1± 4·8 (5)	8·4±5·6 (5)
8	10·9± 6·3 (6)	7·4±6·4 (5)
9	12·4± 4·5 (5)	6·3±3·3 (5)
10	9·1± 4·7 (5)	7·1±3·2 (5)
11	17·5±12·5 (5)	13·8±1·0 (5)
12	14·0± 2·5 (3)	2·5±0·4 (3)
13	7·9± 4·7 (5)	3·3±1·4 (5)
14*	13·3± 4·4 (3)	3·3±1·0 (3)

The estimates of kinin on the rat duodenum were fairly consistently lower than on the uterus, though in one subject (3) the mean was higher. In three subjects (12, 13 and 14) the values were markedly lower on the duodenum. The differences suggested that there is a substance in urine which interfered with one or both assays, and that it was excreted to a much greater extent in some individuals. The differences were small compared

to those occurring with urine which had not passed through alumina. In general, variations were in the same direction on the two tissues. In further analysis of the results only those on the uterus will be discussed, though in most cases specimens were assayed on both tissues.

The estimates of kinin excretion were remarkably constant. There was a less than threefold difference between the means at the extremes of the range (6.5 and 17.5) in 14 subjects. The individual values varied from 2.6 to 38.0 units/min., but in 205 specimens tested only 3 were lower than 3 units/min. and 4 were higher than 30 units/min. The two female subjects (2 and 14) excreted kinin at similar rates to the males.

The Effect of Urinary Output on Kinin Excretion

The early experiments showed that the concentration of kinin in urine was roughly proportional to the concentration of the urine itself. Thus the urinary excretion of kinin has been expressed as units/min. The results in Table V show that the rate of kinin excretion was independent of the urinary output over the range 0.26 to 18 ml./min. In these experiments adjustments to the rate of urine formation (when required) were made by varying the fluid intake.

TABLE V

THE URINARY EXCRETION OF KININ (UNITS/MIN.) AT DIFFERENT RATES OF URINARY OUTPUT (14 SUBJECTS), ESTIMATED ON THE RAT UTERUS

The numbers of specimens studied are given within brackets.

Urinary Output (ml./min.)	Mean±s.e.
0·26 – 0·50	9·8±6·4 (36)
0·51 – 1·00	12·1±5·1 (30)
1·01 – 2·00	12·3±6·6 (47)
2·01 –18·00	10·9±5·1 (19)

The Effect of Urinary pH on Kinin Excretion

It was possible that kinin would be excreted at different rates depending upon the *p*H of the urine. The results obtained with 14 subjects were analysed and are shown in Table VI. The excretion rate of kinin was similar at each *p*H value.

TABLE VI

THE URINARY EXCRETION OF KININ (UNITS/MIN. AT DIFFERENT URINARY *p*H VALUES IN 14 SUBJECTS ESTIMATED ON THE RAT UTERUS

The numbers of specimens studied are given within brackets.

*p*H	Mean±s.e.
5·5	11·9±8·9 (16)
5·8	11·4±6·5 (42)
6·1	13·9±3·7 (13)
6·4	13·3±6·5 (8)
6·7	11·3±4·4 (8)
7·0	12·2±6·0 (6)
7·3	10·9±4·1 (12)

Diurnal Variation of Kinin Excretion

The mean urinary excretion of kinin in 13 overnight specimens was 10.3 (±4.2 s.e.) and in 13 control specimens collected during the day 12.0 (±4.6) units/min. All specimens were obtained from the same subject. These results provide no evidence of any diurnal variation.

It was observed that 3 overnight specimens, collected while the subject was taking sodium bicarbonate orally (urine *p*H 7.3), all contained small amounts of kinin (4.4, 2.6 and 5.3 units/min.). Day specimens which were *p*H 7.3 but were in the bladder for shorter periods did not have these low values.

The Effect of Food on Kinin Excretion

Since kinin is released during functional vasodilatation of the salivary glands (Hilton and Lewis, 1955, 1956), it was possible that the salivation occurring at meals might be associated with increased kinin excretion in the urine. In these experiments meals (usually 2 or 3 course) were not designed specially to promote excessive salivation. The bladder was emptied before the meal and urine was collected 1 to 2 hr. later. Urine specimens were collected over similar (fasting) periods 4 to 6 hr. after a full meal. The mean excretion of kinin in 10 urine specimens collected after meals was 10.9 (±4.2 s.e.) and in the 10 control specimens 13.4 (±8.5) units/min. All specimens were obtained from the same subject. These results showed that kinin was excreted at similar rates during the meal and control (fasting) periods.

The Effect of Sweating on Kinin Excretion

Fox and Hilton (1958) demonstrated the formation of plasma kinin during functional vasodilatation of the sweat glands. In view of this, the effect of sweating on urinary kinin excretion has been investigated. Sweating has been induced by pilocarpine and by heat.

Pilocarpine nitrate (9 mg.) was injected subcutaneously. After 2 min. facial flushing occurred and was quickly followed by profuse salivation and considerable sweating. The symptoms lasted 30 to 40 min. Urine was collected over the 1 to 2 hr. period before the injection and for a similar period after the injection. Three experiments were carried out on one subject. The mean urinary excretion of kinin was 12.1 (±6.0) units/min. before pilocarpine and 16.4 (±7.2) units/min. after a subcutaneous injection of 9 mg. of pilocarpine nitrate. Pilocarpine thus did not produce much change in the kinin excretion rate, although in

each experiment the value during the test period was slightly higher.

One experiment was performed in which sweating was induced by heat. The lower half of the subject was immersed in a hot bath (40 to 43°) for 1 hr. His oral temperature was 38°. To maintain the urinary output 150 ml. warm tea was drunk during the experiment. The excretion of kinin during the experimental period (21 units/min.) was little greater than the rate during the preceding control period (20 units/min.).

Discussion

The Sensitivity and Specificity of the Method

Since the rat duodenum and the rat uterus are so sensitive, only small quantities of kinin are necessary for adequate parallel assays. This is of particular importance for urinary estimations where the kinin concentration is relatively low.

A second advantage of this method is its specificity for kinins. Vasopressin and oxytocin were the only other substances found to be qualitatively similar on the two tissues, but their action on the duodenum required very high doses. In practice these peptides would be distinguished very easily by their much more powerful oxytocic action, and therefore by the lack of agreement between assays on the two tissues.

Biologically, the kinins are more closely related to substance P than to any other known peptide. Both are vasodilators and both stimulate plain muscle. However, the two peptides have opposite actions on the rat duodenum. Substance P produced a slow contraction, an observation which confirms that of Pernow (1953). The rat duodenum may prove to be a useful qualitative test object for distinguishing between these two peptides, but its sensitivity to substance P is low.

Angiotensin may have an inhibitory action on the duodenum in higher doses than those used in this investigation, but the index of discrimination is sufficiently great for it to be distinguished easily from kinin.

The results with bradykinin and urinary kinin are in agreement with more extensive parallel assays and it has been suggested that all these kinins may contain the same active principle (Gaddum and Horton, 1959). However, if the active principles are different, this method will not discriminate between them.

Wasp kinin (Jacques and Schachter, 1954; Schachter and Thain, 1954) and the plasma kinins are very similar; but wasp kinin is inactivated by trypsin and can be separated from the plasma kinins by paper chromatography (Holdstock *et al.*, 1957). They could not be distinguished by parallel assays on the rat uterus and the rat duodenum in the present investigation. If the active principles in wasp kinin and plasma kinin are different, this failure to discriminate is most significant. It suggests that the method described is unable to distinguish between closely allied but chemically different kinins.

Substances which act upon the rat uterus and the rat duodenum may be divided into four groups according to their effects: (1) stimulation of both tissues (namely, substance P), (2) inhibition of both tissues (such as adrenaline), (3) inhibition of the uterus and stimulation of the duodenum (for example, histamine), and (4) stimulation of the uterus and inhibition of the duodenum (like the kinins). Of the substances investigated, only vasopressin and oxytocin fall into the same group as the kinins, and they are easily distinguished. It follows therefore that if an active tissue extract is assayed in parallel on the rat uterus and the rat duodenum using plasma kinin as the standard, and the results agree, it is good evidence that the active principle is really a kinin.

Since it is conceivable, though improbable, that such agreement would occur if the unknown contained a mixture of say 5-hydroxytryptamine and adrenaline, the specificity can be confirmed by showing that the activity is destroyed by chymotrypsin incubation.

Interfering Substances

Biological estimates of the potency of an extract may be modified by the presence of impurities. These may have either an additive or an antagonistic action on the active principle being estimated. The qualitative difference in the responses of the rat uterus and the rat duodenum to kinin is particularly valuable in the detection of such interfering substances.

If the kinin extract contains an inhibitory substance such as adrenaline, its potency measured by the rat uterus will be low, because the kinin in the extract will be partially inhibited. On the other hand, the potency measured by the rat duodenum will be high, because both kinin and the impurity will inhibit that tissue and their effects will be additive. Thus, the discrepancy between results on these two tissues will tend to be more marked than with a pair of tissues which responded in a qualitatively similar manner, and smaller amounts of impurity will be detected.

One such impurity was soon detected when crude urine was used for the assays. This inhibitory substance could be removed by

alumina adsorption. However, there was still a slight discrepancy between the results on the two tissues, indicating that some other interfering substance was present.

Stability of Kinin in Urine

The loss of activity of urine specimens at room temperature is of great significance from a practical viewpoint. If a true figure for kinin excretion is to be obtained the urine must either be assayed within a short time of collection, or steps must be taken to prevent the inactivation. This generally precludes the possibility of estimating it in 24 hr. samples as collected clinically. The experiments in which urine specimens were protected from inactivation by *p*H adjustment or by previous boiling strongly suggest that an enzyme is responsible for much of the loss of activity in crude urine, confirming the findings of previous workers (Frey *et al.*, 1950). The action of the enzyme was prevented most conveniently by freezing the urine samples as soon as possible after collection.

The Excretion of Kinin in Human Urine

The results show that the rate of urinary kinin excretion is fairly constant. Conditions under which kinin is known to be formed in increased amounts were not associated with much change in the urinary kinin output. This suggests that the kinin released during glandular activity must be destroyed rapidly, before the blood levels rise sufficiently to alter the urinary content. Such inactivation by plasma occurs very readily *in vitro*, and is thus to be excreted *in vivo*. In addition, since a kininase is present in urine, some kinin destruction may occur in the bladder.

The question of the origin of urinary kinin remains unsettled. It may represent the renal clearance of kinin from the plasma. Human plasma which has been collected with precautions to prevent glass activation (pre-active plasma) does contain small amounts of kinin (Armstrong *et al.*, 1957) and this may represent the normal plasma level. On the other hand, it is possible that the kinin in urine is formed locally in the kidney and that it is a mediator of renal vasodilatation similar to its function in the salivary and sweat glands.

I am very grateful to Professor J. H. Gaddum, F.R.S., for his encouragement and advice during this investigation. The work reported in this paper was done during the tenure of a Graduate Research Scholarship at the University of Edinburgh, and forms part of a Thesis submitted for the degree of Ph.D.

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THE BIOLOGICAL ACTIVITY OF A NEW ANALOGUE OF OXYTOCIN IN WHICH THE TYROSYL GROUP IS REPLACED BY PHENYLALANYL

BY

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A new synthetic analogue of oxytocin with a phenylalanyl group instead of a tyrosyl group has been investigated. The most prominent biological properties of this peptide were its oxytocic activity on the rat or cat uterus *in vivo* and on milk-ejection pressure in the rabbit. Another remarkable feature was the striking difference in pressor activity measured in spinal cats and in anaesthetized rats. The presence of the phenylalanyl group in the molecule in the above-mentioned position did not increase the antidiuretic potency in unanaesthetized rats. The authors propose a nomenclature for the peptides related to oxytocin and vasopressin.

As chemical and biological work on peptides related to oxytocin and vasopressin progresses, the need for a simple and clear nomenclature in this field becomes increasingly greater. We should therefore like to propose a nomenclature along the lines of that already in use for polypeptides of the hypertensin type (see, for example, Rittel, Iselin, Kappeler, Riniker, and Schwyzer, 1957). The amino-acid groups in the oxytocin molecule would be numbered as follows:

1 2 3 4 5 6 7 8 9
CyS-Tyr-Ileu-GluNH$_2$-AspNH$_2$-CyS-Pro-Leu-GlyNH$_2$

The number of the group which the new amino-acid replaces would be indicated as a superscript to the new group. The analogue dealt with in this paper would therefore be named phenylalanyl^2-oxytocin. The oxytocin analogues which have so far been investigated (Rudinger, Honzl, and Zaoral, 1956; Boissonnas, Guttmann, Jaquenoud, Waller, Konzett, and Berde, 1956a; Berde, Doepfner, and Konzett, 1957; Katsoyannis, 1957; Ressler and du Vigneaud, 1957; Ressler and Rachele, 1958) would then be specified as follows: phenylalanyl^3-oxytocin (this is also known as oxypressin or the P-analogue); leucyl^3-oxytocin (also known as the L-analogue); valyl^3-oxytocin (also known as valyl-oxytocin or the V-analogue); glutaminyl^5-oxytocin (also known as the G-analogue); and isoglutaminyl^4-oxytocin (also known as isoglutamine isomer of oxytocin or isoglutamine-oxytocin). With this nomenclature, the vasopressins could also be described as oxytocin analogues, namely arginine-vasopressin as phenylalanyl^3-arginyl^8-oxytocin and lysine-vasopressin as phenylalanyl^3-lysyl^8-oxytocin. In the case of the synthetic higher homologues the term " homo " would precede the new amino-acid group and would have as superscripts the numbers of those two groups between which the new one has been inserted, for example, homotyrosyl$^{2:3}$-oxytocin (Guttmann, Jaquenoud, Boissonnas, Konzett, and Berde, 1957; Konzett, 1957). In the present paper the above nomenclature will be used.

Recent work in this laboratory (Boissonnas *et al.*, 1956a; Berde *et al.*, 1957; Berde and Cerletti, 1957; Guttmann *et al.*, 1957; Konzett, 1957; Berde and Cerletti, 1958) and elsewhere (Katsoyannis, 1957; Ressler and du Vigneaud, 1957; Ressler and Rachele, 1958) on the biological activity of synthetic analogues of oxytocin has revealed that relatively small chemical changes in the oxytocin molecule may lead to a decrease, increase, or modification of its characteristic effects.

If, for example, the asparaginyl group in the oxytocin molecule is replaced by a glutaminyl group (glutaminyl^5-oxytocin), the physiological and pharmacological actions peculiar to the oxytocic hormone of the pituitary are absent (Berde *et al.*, 1957). The same holds true if an isoglutaminyl group is substituted for the glutaminyl group (isoglutaminyl^4 - oxytocin) (Ressler and du Vigneaud, 1957; Ressler and Rachele, 1958). The biological activity of the

oxytocin analogue which contains a leucyl group instead of the isoleucyl group (leucyl^3-oxytocin) is rather modest (Berde *et al.*, 1957). Nevertheless, this compound exerts all the typical actions of oxytocin, albeit on a reduced scale.

However, replacing the isoleucyl group of the oxytocin molecule by a valyl group (valyl^3-oxytocin) brings a striking increase of the *in vivo* activity on the uterus and on the mammary gland, together with a decrease of the pressor and antidiuretic potency (Boissonnas *et al.*, 1956a; Berde *et al.*, 1957). This means that the uterus-stimulating and milk-ejecting effects of valyl^3-oxytocin are even more specific than those of the natural oxytocic hormone.

The cyclic octapeptide possessing a phenylalanyl group in the position where oxytocin contains an isoleucyl group (phenylalanyl^3-oxytocin) is interesting because this compound is, chemically, an intermediate between oxytocin and vasopressin. This fact is also borne out by pharmacological studies (Berde *et al.*, 1957; Katsoyannis, 1957): both the qualities of oxytocin (uterus-stimulating, milk-ejecting, and avian depressor activity) and of vasopressin (pressor and antidiuretic activity) are exhibited with remarkably high potency.

Phenylalanine is found in the vasopressin obtained both from cattle and pigs. It could therefore be assumed that the phenylalanyl group *per se* is mainly responsible for the effects peculiar to vasopressin. The substitution of a phenylalanyl group for any amino-acid group in oxytocin would then cause biological properties to shift towards those of vasopressin. On the other hand, it could be argued that, in order to exert such a decisive influence on the pharmacological character of the cyclic octapeptide, the phenylalanyl group would have to occupy the same position which it occupies in the vasopressin molecule, where it is located in the ring between the tyrosyl and the glutaminyl group. The pharmacological study of a synthetic analogue of oxytocin with a phenylalanyl group in some other position was therefore of considerable interest.

Such an analogue of oxytocin with a phenylalanyl group substituted for the tyrosyl group, phenylalanyl^2-oxytocin (Table I), has recently been synthesized in our Department of Pharmaceutical Chemistry by P. A. Jaquenoud in 1958 using the same method of synthesis as previously used for the synthesis of oxytocin (Boissonnas, Guttmann, Jaquenoud, and Waller, 1955): To obtain the cyclic octapeptide, 20 mg. of the corresponding nonapeptide (Boissonnas, Guttmann, Jaquenoud, and Waller, 1956b) was dissolved in 5 ml. of liquid ammonia, and reduced with sodium (du Vigneaud, Ressler, Swan, Roberts, and Katsoyannis, 1954). Ammonium chloride (5 mg.) was added; the ammonia was evaporated and the residue added to 100 ml. of water, the *p*H adjusted to 6.8, CO_2-free air bubbled through the solution for 2 hr., and the *p*H adjusted to 4.5. Saline was used for further dilution.

TABLE I

THE SEQUENCE OF THE AMINO ACIDS IN OXYTOCIN AND IN PHENYLALANYL^2-OXYTOCIN

CyS = L-cysteinyl; Tyr = L-tyrosyl; Ileu = L-isoleucyl; Glu(NH_2) = L-glutaminyl; Asp(NH_2) = L-asparaginyl; Pro = L-prolyl; Leu = L-leucyl; Gly(NH_2) = glycinamide; Phe = phenylalanyl.

Synthetic oxytocin	CyS-Tyr-Ileu-Glu(NH_2)-Asp(NH_2)-CyS-Pro-Leu-Gly(NH_2)
Phenylalanyl^2-oxyto	CyS-Phe-Ileu-Glu(NH_2)-Asp(NH_2)-CyS-Pro-Leu-Gly(NH_2)

METHODS

Six tests were used to detect and measure pharmacological effects peculiar to oxytocin. These included the rat uterus *in vitro* (Holton, 1948) and *in vivo* (Berde *et al.*, 1957), as well as the cat uterus *in vivo* (Berde *et al.*, 1957) for the uterus-stimulating (oxytocic) activity; the mammary gland of lactating rabbits (Van Dyke, Adamsons, and Engel, 1955; Berde and Cerletti, 1957) for the milk-ejecting potency; the blood pressure of chickens (Coon, 1939; Thompson, 1944) for the avian depressor effect; and the diuresis of saline-loaded rats (Lipschitz, Hadidian, and Kerpcsar, 1943) for the diuretic effect.

Four tests were used to demonstrate and measure biological activities characteristic of vasopressin. These were the blood pressure of spinal cats and the blood pressure of rats pre-treated with dibenamine and heparin, according to Dekanski (1952), for the pressor potency; the rabbit ileum *in vitro* for the effect on the smooth muscle of the intestine; and the diuresis of water-loaded rats (Burn, 1931; Burn, Finney, and Goodwin, 1950) for the antidiuretic quality. In the first three of these tests, the effectiveness of the unknown preparation was estimated by bracketing doses of the unknown with doses of the standard. The results obtained did not permit accurate determination of the limits of error. To measure the antidiuretic activity a four-point assay design (Schild, 1942) was employed.

The Third International Standard for Posterior Pituitary (the Third International Standard for Oxytocic, Vasopressor, and Antidiuretic Substances) was used for comparison in all but one test. In the diuresis experiments on saline-loaded rats, synthetic oxytocin, also

known as Syntocinon (Boissonnas *et al.*, 1955; Konzett, Berde, and Cerletti, 1956), was used for this purpose.

Results

The results are summarized in Table II. The values obtained with the two conventional methods for the bioassay of oxytocin (isolated rat uterus and chicken blood pressure) differed,

Table II

ACTIVITY IN VARIOUS TESTS OF 1 ML. OF SOLUTION OF PHENYLALANYL[2]-OXYTOCIN IN UNITS OF THE INTERNATIONAL STANDARD (THIRD INTERNATIONAL STANDARD FOR OXYTOCIC, VASOPRESSOR, AND ANTIDIURETIC SUBSTANCES)

Synthetic oxytocin was used in the diuretic test as the basis of comparison. Standard errors shown in parentheses.

Isolated rat uterus	1·6 (±0·16)
Chicken blood pressure	2·6 (±0·24)
Isolated rabbit uterus	1·6
Rat uterus *in vivo*	3·7 (±0·64)
Cat " " "	10·7 (±2·2)
Milk-ejection pressure in rabbit	6·2 (±0·64)
Pressor activity in spinal cat	0·16
" " " rat	0·016
Isolated rabbit intestine	0·016
Antidiuretic activity	0·024 (±0·006)
Diuretic "	10·7 (±6·7)

whereas the activity on the isolated rat uterus and the isolated rabbit uterus was identical. The activities on the uterus *in vivo* of rats and cats were considerably higher than that on the rat uterus *in vitro*, as was the action on the milk-ejection pressure in the rabbit mammary gland.

There was a quite remarkable difference between the pressor activity on the spinal cat and the pressor activity on the anaesthetized rat. The potency on the isolated rabbit intestine resembled that on the blood pressure of the rat rather than that on the blood pressure of the spinal cat. The antidiuretic activity was low, whereas the diuretic activity was remarkably high.

It is quite clear from these findings that the potency of the new analogue, expressed in units of the International Standard for Posterior Pituitary, depends on the biological indicator used for the assay. As the relative activities in the different tests may be of special interest, these are given in Table III, the oxytocic effect on the rat uterus *in vitro* being taken arbitrarily as 100. To facilitate a comparison of the new analogue with oxytocin and some interesting oxytocin analogues which have been previously described, Table III also gives the corresponding relative values for synthetic oxytocin, for valyl[3]-oxytocin, and for phenylalanyl[3]-oxytocin, as established in this laboratory (Konzett *et al.*, 1956; Berde *et al.*, 1957).

Table III

RELATIVE ACTIVITIES IN VARIOUS TESTS OF SYNTHETIC OXYTOCIN AND OF SOME ANALOGUES OF OXYTOCIN

The potency of each compound as measured on the isolated rat uterus is arbitrarily taken as 100. Diuretic potency of synthetic oxytocin arbitrarily taken as 100 and used as standard of comparison for the diuretic activity of phenylalanyl[2]-oxytocin.

Test	Phenyl-alanyl[2]-oxytocin	Synthetic Oxytocin	Valyl[3]-oxytocin	Phenyl-alanyl[3]-oxytocin
Isolated rat uterus	100	100	100	100
Chicken blood pressure	165	101	114	81
Isolated rabbit uterus	100	100		
Rat uterus *in vivo*	230	100	300	
Cat " " "	670	130	600	89
Milk-ejection pressure in rabbit	390	104	536	215
Pressor activity in spinal cat	10	0·9	0·5	19
Pressor activity in rat	1	1·1		
Antidiuretic activity	1·5	1·2	1·4	104
Diuretic activity	670	100	71	

Discussion

Comparison of the relative potency of the new oxytocin analogue having a phenylalanyl group instead of the tyrosyl group with that of oxytocin and of other previously investigated analogues of oxytocin yields some remarkable facts.

The activity of phenylalanyl[2]-oxytocin in the two conventional bioassays for oxytocin (the rat uterus *in vitro* and the chicken blood pressure) was discrepant, whereas the corresponding values for oxytocin, valyl[3]-oxytocin and phenylalanyl[3]-oxytocin were in good agreement. However, the cyclic disulphide ring of oxytocin (which has no prolyl - leucyl - glycinamide side - chain) also exhibited some discrepancy when assayed in the conventional way: it exerted an effect on the isolated rat uterus but none on the chicken blood pressure (Ressler, 1956). By contrast, the new analogue exerted a greater effect on the chicken blood pressure than on the isolated rat uterus.

The potency of phenylalanyl[2]-oxytocin measured on the rat and cat uterus *in vivo* and on the rabbit mammary gland was definitely higher than when measured by the methods suggested by the pharmacopoeias (British Pharmacopoeia, 1958; United States Pharmacopeia XV, 1955) for the bioassay of oxytocin (rat uterus *in vitro*, chicken blood pressure). This type of discrepancy has already been observed with valyl[3]-oxytocin, and to some degree with phenylalanyl[3]-oxytocin (Boissonnas *et al.*, 1956a; Berde *et al.*, 1957) and

proved to be of practical importance, as the testing of valyl[3]-oxytocin on the human uterus *in vivo* also gave values higher than those obtained with the methods advised in the pharmacopoeias (Smyth, 1958). The same trend was found with leucyl[3]-oxytocin (Berde *et al.*, 1957), although this peptide was not tested on the rat uterus *in vivo*. As yet, no synthetic analogue of oxytocin has been found to show a discrepancy in the opposite sense, that is, an activity in the official bioassay methods higher than that on the uterus *in vivo* and on the lactating mammary gland. No explanation for this has yet been found.

The pressor activity of the new oxytocin analogue on spinal cats was much greater than that of oxytocin and valyl[3]-oxytocin, but smaller than that of phenylalanyl[3]-oxytocin. Thus, the presence of a phenylalanyl group in the cyclic part of the octapeptide increased the pressor activity of both compounds in spinal cats. It was very surprising that the activity of the new analogue on the blood pressure of anaesthetized rats was so much smaller (about 1/10) than that on the blood pressure of spinal cats. This was noteworthy, as the pressor activity of pituitary extracts from ox, cat, and rat was found to be practically identical by the rat and cat methods (Landgrebe, Macauley, and Waring, 1946). Furthermore, the results of our assays of the pressor potency of synthetic oxytocin and synthetic lysine-vasopressin on cats and rats showed close agreement.

Whereas the substitution of a phenylalanyl group for the isoleucyl group produced a tremendous increase of antidiuretic activity (from 1.2 to 104), the substitution of a phenylalanyl group for the tyrosyl group barely increased the antidiuretic activity (from 1.2 to 1.5). As far as water excretion was concerned, the presence of a phenylalanyl group instead of the tyrosyl group did not produce the appearance of vasopressin-like properties.

From the results reported it may be concluded that the position at which a certain amino-acid was substituted is very important for the biological activity of oxytocin analogues. The introduction of the same amino acid at various positions in the molecule may result in octapeptides with very marked differences in action, as a comparison of phenylalanyl[2]-oxytocin with phenylalanyl[3]-oxytocin showed. Whereas phenylalanyl[3] - oxytocin was biologically an intermediate between oxytocin and vasopressin, the oxytocin-like qualities of phenylalanyl[2]-oxytocin were paramount, especially *in vivo* (rat uterus, cat uterus, milk-ejection pressure).

It should, however, be borne in mind that in replacing one amino acid by another the actual chemical changes of the molecule may be small. Phenylalanyl[2]-oxytocin contains only one hydroxyl group less than oxytocin and valyl[3]-oxytocin contains only one CH_3 group less than oxytocin.

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TRACER STUDIES OF THE DISTRIBUTION AND TRYPANOCIDAL ACTION OF STILBAMIDINE IN RATS

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[^{14}C]Stilbamidine was used to study the distribution of the drug in the organs and tissues of rats following intravenous injection. The prophylactic action of stilbamidine was shown to depend upon the unchanged drug retained in tissues, especially the liver. Only the parent drug was extracted from trypanosomes when an infection was treated during the acute phase, as shown by the use of the fluorescent properties of stilbamidine in conjunction with scanning and chromatographic techniques. The action of stilbamidine on trypanosomes is therefore a direct one. A method for the synthesis of [^{14}C]stilbamidine in much improved yield is also described.

Reid and Weaver (1951) measured the distribution of radioactivity in tissues of mice at intervals after administration of [^{14}C]stilbamidine. In the experiments to be described similar measurements were made in rats and chromatographic methods were used to determine the chemical nature of the radioactive substances. The same methods enabled us to determine, more accurately than earlier methods allowed, the uptake of the drug by trypanosomes in the infected rat, and to indicate the nature of the compound responsible for prophylaxis.

Laveran and Mesnil (1904) observed that trypanosomes which had become sluggish or immobile in citrated blood recovered their motility on addition of fresh blood or serum. Biot, Biot and Richard (1911) found that glucose increased the survival time of *T. lewisi in vitro*. The need for glucose was reported for a number of other species of trypanosomes. This led to the search for trypanocidal agents with hypoglycaemic properties. Two groups of workers, von Jancsó and von Jancsó (1935) and Schern and Artagaveytia-Allende (1936), discovered that guanidine and certain derivatives possessed trypanocidal properties *in vivo*, the most active being decamethylenediguanidine (Synthalin). King, Lourie, and Yorke (1937) examined many compounds related to Synthalin, and discovered that certain diamidines had marked trypanocidal action *in vivo* and *in vitro*. The synthesis of these compounds was exploited by Ashley, Barber, Ewins, Newbery, and Self (1942). The most active substances they discovered were stilbamidine, propamidine, and pentamidine. The pharmacology, mode of action, and therapeutic uses of diamidines have been discussed by Heathcote (1946), Schoenbach and Greenspan (1948), Kirk (1957), and others. These substances have been shown to inhibit the growth of bacteria, protozoa, pathogenic fungi, and, to some extent, neoplastic cells. Stilbamidine and propamidine have proved somewhat toxic, but pentamidine is still widely used as a prophylactic against human trypanosomiasis, and an injection given every 6 months ensures protection from the disease. Kopac (1947) has observed that stilbamidine brings about a general denaturation of the nucleoproteins, but the mode of action of diamidines as a class has not been elucidated.

MATERIALS AND METHODS

Biological Methods

The strain of *Trypanosoma rhodesiense* used was isolated from a patient with sleeping sickness in 1923 and has since been maintained by syringe passage of infected blood in mice. White rats of weight 150 to 200 g. were used in the present experiments. Each animal was given 2 to 3 mg. of [^{14}C]stilbamidine (2.5 to 3.8 μC.) in distilled water by a tail vein or, more rarely, intraperitoneally. Specimens of blood, liver, kidney, lung, spleen, heart, intestine, and faeces were freeze-dried, then powdered and plated for measurement of radioactivity on polythene discs with an area of 1 cm.^{2} (Popják, 1950). Urine samples were directly evaporated on lens tissue on polythene discs. Samples were counted at "infinite thickness" by a thin-mica-window Geiger-Müller counter.

For the study of the uptake of stilbamidine by trypanosomes *in vivo*, infected rats were treated with 2 mg. of the radioactive material (0.69 μC./mg.). After 3 hr. the animals were anaesthetized and bled by cardiac puncture into citrated saline and the parasites isolated as described by Fulton and Spooner (1956). They were washed by resuspension in 10% horse serum in buffered saline (Krebs and Eggleston, 1940) and then centrifuged. After a final wash in saline they were isolated as a white mass free from white cells, then freeze-dried and plated as above. Correction was made for sodium chloride in the suspending liquid. For chromatographic examination the trypanosomes were extracted by grinding with sand mixed with 80% aqueous ethanol containing 1 to 2% acetic acid. In another series of experiments 6 rats were given a dose of 2.0 mg. of [^{14}C]stilbamidine (1.27 μC./mg.), and after certain intervals varying from 1 to 21 days an animal was heavily infected with trypanosomes. 24 hr. later trypanosomes had multiplied, the animal was killed, and the parasites were isolated from the blood. On freeze-drying, the radioactivity of the sample was measured.

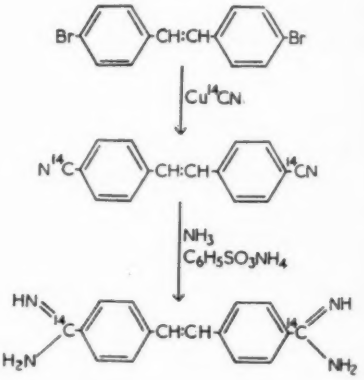
To find whether stilbamidine was metabolized by rat tissue *in vitro*, 10 g. of fresh liver was obtained aseptically and ground up in approximately 32 ml. saline containing 0.2% glucose. The suspension was divided into 3 portions of 10 ml. To one portion was added 1.3 ml. of a [^{14}C]stilbamidine solution containing 1 mg./ml. (activity 1.27 μC./mg.). All 3 portions were incubated for 4 hr. at 37°. A similar amount of stilbamidine was added to the second portion at the end of incubation. To the third portion the same volume of saline was added. Each portion was then centrifuged at 15,000 *g* for 30 min. The supernatant was separated from the deposit and its radioactivity measured. The supernatant portion and extracts from the deposit were also examined chromatographically.

A typical extraction of a tissue was carried out as follows. Wet liver (1.5 g.) was ground with clean sand and stirred with 50 ml. of 1% acetic acid (Lieben and Snapper, 1950) for 20 min. The suspension was centrifuged at 3,000 rev./min. for 30 min. and the clear solution decanted. The residue was re-extracted with 30 ml. of 1% acetic acid as before. The solid residue was separated as far as possible from sand and when dried gave 37.5 counts/min. as against 430 in the original sample. The combined extracts were centrifuged again to remove a further small amount of precipitate and concentrated under reduced pressure to a small volume.

For determination of radioactivity due to stilbamidine in urine, carrier technique was employed. To 20 ml. of urine was added 70 mg. of non-radioactive stilbamidine hydrochloride in 20 ml. water. Stilbamidine sulphate was precipitated by addition of 5 ml. 2N-H_2SO_4. The sulphate was collected by centrifugation and stirred with 5 ml. of N-NaOH; the base was collected by centrifugation and converted by dilute HCl to the hydrochloride which was crystallized three times in the presence of charcoal from dilute HCl. The purified hydrochloride (17.6 mg.) was powdered and plated.

Chemical Methods

A synthesis of [^{14}C]stilbamidine was carried out by Reid and Weaver (1951), but the overall yield based on cyanide (the form in which ^{14}C was introduced) was only 19%. To improve yields, the reaction of 4:4′-dibromostilbene with cuprous cyanide and the preparation of the amidine from the dinitrile were studied in detail with non-radioactive materials. By using a very pure specimen of the dibromo-compound and a 7% excess of cuprous cyanide, an 81% yield based on cyanide was obtained. The conversion to amidine was effected by fusing the dinitrile with ammonium benzenesulphonate (Oxley and Short, 1946; Oxley, Partridge and Short, 1948) in a stream of ammonia to give a 75% yield. The synthesis may be represented thus:



4:4′-Dibromostilbene.—This was prepared from dibenzyl according to Bance, Barber, and Woolman (1943) and formed pearly white crystals after four recrystallizations from glacial acetic acid; m.p. 210 to 212°.

4:4′-Di[^{14}C]cyanostilbene. — Radioactive cuprous cyanide from the Radiochemical Centre, Amersham (27.8 mg., 500 μC.), was mixed with ordinary cuprous cyanide (166 mg.) and pure 4:4′-dibromostilbene (359 mg.). Dry pyridine (0.25 ml.) was added and the mixture heated under reflux for 1 hr. at 220° in an atmosphere of nitrogen. The cooled product was extracted with 12 ml. of hot pyridine, and the solution poured into excess concentrated HCl (20 ml.). The precipitated dicyanostilbene was collected on sintered glass, washed with concentrated HCl, then water, and dried. Crystallization from 7 ml. of pyridine gave a very pale yellow product (202 mg.), m.p. 283 to 286°.

The mother liquid was mixed with ordinary dicyanostilbene (100 mg.), concentrated a little, and cooled. The crystals (107 mg.) were collected and used to prepare stilbamidine of lower radioactivity.

4:4′-Stilbenedi[^{14}C]*amidine* (*Stilbamidine*). — The radioactive dicyanostilbene (202 mg.) was mixed with dry ammonium benezenesulphonate (760 mg.) in a small tube and heated to 275° in a metal bath. A slow stream of dry ammonia was bubbled through the melt for 1½ hr.; after the first 15 min. a solid began to separate from the melt. The cooled product was warmed with water, broken up, and treated, while cooling, with 2N-KOH solution (5 ml.). The crude solid amidine was collected and washed with water on a sintered glass filter. It was dissolved on the filter in 0.1N-HCl (20 to 25 ml.). The small residue (mostly unchanged dicyanostilbene) was added after washing with water to dicyanostilbene of lower radioactivity. The pale yellow solution of the hydrochloride (filtrate and washings) was concentrated to 7 ml. under low pressure and treated with 1 ml. of concentrated HCl. After cooling, the crystals of stilbamidine hydrochloride were collected, washed with a small quantity of 3N-HCl, and dried. The product (242.6 mg., 74%) was crystallized twice from water (3 to 4 ml.) with addition of charcoal to give nearly colourless crystals (160 mg.).

The same product from a similar run of non-radioactive material was analysed after drying in air for 24 hr. (Found: C, 51.66; H, 5.86; N, 14.97; Cl, 18.4; required for $C_{16}H_{16}N_4$, 2HCl, $2H_2O$: C, 51.47; H, 5.89; N, 15.02; Cl, 19.01%.)

The dicyanostilbene of lower radioactivity (107 mg.) was converted to stilbamidine by heating with ammonium benzenesulphonate (500 mg.) as before. The solution of the hydrochloride was mixed with the mother liquors of the previous run, then concentrated and crystallized. Colourless material (75 mg.) was obtained on recrystallization from water with addition of charcoal. Counting was done after dilution of each sample with several hundred times its weight of non-radioactive stilbamidine hydrochloride followed by recrystallization. The specific activities found for the two (undiluted) samples were: Sample I, 1.27 μC./mg., and sample II, 0.70 μC./mg.

Chromatographic Methods

Paper chromatographic methods have not previously been reported for the separation of stilbamidine and the amides derived from it. Ascending chromatography in pyridine-*iso*amyl alcohol-water (7:7:6 by volume) was the most satisfactory system of several tried. The chromatography was run for 18 to 24 hr. and the spots were detected on the dried paper by ultraviolet light.

The R_F values for some of the compounds were: stilbamidine, 0.28; 4-amidino-4′-carbamoylstilbene, 0.51; 4:4′-dicarbamoylstilbene, 0.71.

The chromatograms were scanned for radioactivity by an instrument designed to count every square cm. for 1 min. and record the results automatically (Piper and Arnstein, 1956). Fluorescent non-radioactive spots were frequently observed, but no definite indication of non-fluorescent radioactive spots was found. At the start of this work confusion was caused by the presence of a well-defined radioactive spot a little in front of that due to stilbamidine which developed fluorescence gradually under ultraviolet illumination, and was due to the *cis*-form of stilbamidine which appears when stilbamidine is exposed to light but not otherwise. This was confirmed by using an authentic specimen of the *cis*-form kindly supplied by Dr. J. N. Ashley of May and Baker Ltd. Subsequently light was excluded during chromatography and no *cis*-stilbamidine was then found.

RESULTS

Distribution and Excretion of Drug.—The distribution of radioactivity in different organs and blood of the white rat is shown in Table I.

TABLE I

DISTRIBUTION OF [^{14}C]STILBAMIDINE IN RATS

The percentage of total injected radioactivity in the whole organ is given in the first column under each organ. The second numeral is the number of counts recorded/min. by a dried sample at infinite thickness on a 1 cm.^{2} disc and is a measure of the specific activity of the organ.

Rat	Dose; Route; Radioactivity	Interval After Dosing	Liver		Kidneys		Lungs		Spleen		Heart		Intestines		Serum	
			%	Counts	%	Counts	%	Counts	%	Counts	%	Counts	%	Counts	%	Counts
1	2 mg.; i.v. (2·55 μC.)	2 hr.	24·6	423	7·59	594	1·41	75	0·79	130	0·12	24	2·59	33	0·04	4·0
2	3 mg.; i.v. (3·8 μC.)	24 ,,	18·8	272	8·5	795	0·51	36·5	0·49	79	0·05	11·0	1·25	21·4	—	—
3	,, ,,	3 days	19·3	285	5·03	327	0·96	81·7	0·32	48	0·13	24·6	1·28	20·0	—	—
4	2·7 mg.; i.p. (3·4 μC.)	3 ,,	11·0	191	4·8	415	0·97	82·7	0·41	59·6	0·10	13·1	—	—	—	—
5	3 mg.; i.v. (3·8 μC.)	7 ,,	4·75	64·6	0·87	70	0·34	41·2	0·21	23·0	0·07	12·0	0·69	6·9	—	—
6	,, ,,	14 ,,	6·61	132	0·76	80	0·36	60·5	0·19	24·2	0·10	16·3	1·61	25·5	—	—
7	,, ,,	21 ,,	1·18	19·4	0·56	56·7	0·37	48·0	0·18	24·0	0·07	15	0·55	8·8	—	—
8	,, ,,	28 ,,	2·78	48·5	0·71	71·7	0·28	39·4	0·066	9·6	0·08	15·2	1·23	18·4	—	—
9	,, ,,	50 ,,	0·39	6·2	0·44	43·2	0·17	22·0	0·11	12·7	0·06	9·1	0·51	5·6	—	—
10	,, ,,	165 ,,	0·16	2·3	0·30	25·9	0·12	9·4	0·02	2·4	0·10	18·0	—	—	—	—
11	,, ,,	230 ,,	0·16	2·2	0·18	13·8	0·17	13·0	—	—	0·05	10·0	—	—	—	—
12	,, ,,	280 ,,	—	—	0·18	14·4	0·07	5·3	—	—	0·04	7·7	—	—	—	—

Previously Reid and Weaver (1951) had studied the same problem in mice while investigating the possibility of selective irradiation of plasma cells by administration of the drug to patients with multiple myeloma, and our results agree qualitatively with theirs. Probably because of the fact that only one animal was sacrificed at the end of each interval after administration of drug in these experiments the fall in radioactivity was not strictly correlated with the time of examination. It will be noted that the liver and kidney took up respectively 25 and 7.5% of the injected dose within the first 2 hr., the highest concentration (specific activity) being in the kidneys. In about 50 days most of the drug had been eliminated as indicated by the fact that radioactivity was below 0.5% of that originally present in liver and kidneys. Although lung, spleen, and heart took up relatively little drug they retained activity longer than some other organs. The radioactivity of serum corresponded to about 0.25 μg. of the drug/ml. 2 hr. after its administration and thereafter activity fell to zero. The red blood cells were also found to have no appreciable activity after 4 hr., and this confirms the earlier work of Fulton and Goodwin (1945). The radioactivity excreted in urine and faeces during the first 24 hr. following drug administration was 2.5 and 6.1% respectively of the injected dose.

Chemical Nature of Retained and Excreted Radioactivity.—Urine collected over 8 days from 12 rats each of which received 3 mg. (3.8 μC.) of stilbamidine was assayed for radioactivity, and the proportion of radioactivity present as stilbamidine was found by the carrier technique (see Methods) to be 68%. Chromatography of the urine showed minor proportions of the two amides, 4-amidino-4′-carbamoylstilbene and 4:4′-dicarbamoylstilbene, known to be formed from stilbamidine in solution by hydrolysis (Henry, 1948; Fulton and Goodwin, 1949). Substantially all the radioactivity in urine was extractable by chloroform or *iso*amyl alcohol after addition of alkali, but extractions of acidified urine by the same solvents removed only traces of radioactivity. Thus almost all the radioactivity was present in basic substances.

Extracts were made of liver and kidney from the series of injected animals and chromatograms of the extracts were scanned for fluorescence and for radioactivity. This study showed that even after long periods the radioactivity remaining in these tissues was due largely to unaltered stilbamidine; traces of the related amides were also present. Since extraction of radioactivity from tissue was virtually complete, and no non-fluorescent radioactive spots were detected, the possibility is slight that significant amounts of another metabolite were overlooked.

Chromatography of extracts of faeces showed stilbamidine accompanied by rather higher proportions of the amides than were found in tissues.

TABLE II

ABSORPTION OF PROPHYLACTIC DOSES OF STILBAMIDINE BY TRYPANOSOMES

Interval between Drug Administration and Infection (Days)	Radioactivity of Isolated Trypanosomes (Counts/Min. at Infinite Thickness)
1	13·5
2	16·0
4	8·1
7	6·4
14	3·0
21	Slightly above background

Prophylactic Experiments and Uptake of Drug by Trypanosomes.—The results of experiments designed to show the mode of action of stilbamidine in prophylactic experiments are given in Table II. It had been found previously that the prophylactic activity of the drug lasted about three weeks in mice treated with the maximum tolerated dose of non-radioactive material (Fulton, 1944). It is clear from the present results that appreciable quantities of drug are taken up by the parasites during the first few days after administration, but not after 21 days when the amount of drug stored in the liver was only 1 to 2% of the initial dose. This fact taken in conjunction with the absence of metabolites suggests that the prophylactic action of stilbamidine is the result of a direct action by the unchanged drug stored in the body. Fulton and Grant (1955, 1956) came to the same conclusion when they used non-radioactive stilbamidine. The incubation of stilbamidine with rat liver in the present series of experiments also failed to indicate conversion of the drug to a metabolite. In the same way, when a rat infected with trypanosomes was treated with the radioactive drug, and the parasites, which rapidly absorb stilbamidine, were harvested after 4 hr. and extracted with a suitable solvent, no substance other than the parent drug was detected by chromatographic and scanning techniques. The results in the present experiments having been obtained with more accurate techniques indicate a somewhat higher uptake of drug by the trypanosomes than was previously reported by the above workers.

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INCREASED URINARY EXCRETION OF ADRENALINE AND NORADRENALINE AFTER PHENOXYBENZAMINE

BY

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In dogs anaesthetized with pentobarbitone sodium phenoxybenzamine greatly increased the urinary excretion of adrenaline and noradrenaline. This was associated with a transient increase in the volume of urine excreted and a fall in blood pressure. Hexamethonium prevented the effects on the urinary amines and on the blood pressure. Methacholine hypotension induced an increased adrenaline excretion, but no change in noradrenaline. The excretion of adrenaline released following ganglionic stimulation by acetylcholine did not appear to be affected by phenoxybenzamine, but that of noradrenaline was increased. During infusions of adrenaline or noradrenaline, phenoxybenzamine increased the excretion of both adrenaline and noradrenaline.

In earlier experiments (Benfey, Mazurkiewicz, and Melville, 1958a and b) it was shown that in dogs anaesthetized with phenobarbitone there was an increased urinary recovery of injected noradrenaline when various antisympathetico-mimetic agents were given (piperoxan, Hydergin, ergotoxine, chlorpromazine). As a continuation of these studies, the effects of phenoxybenzamine upon the urinary excretion of adrenaline and noradrenaline were investigated. It was observed that this agent led to a prolonged and increased excretion of both adrenaline and noradrenaline, and the experiments now described were undertaken to investigate this. Preliminary reports of this work have already appeared (Benfey, Ledoux, and Melville, 1958; Ledoux, Melville, and Benfey, 1958).

METHODS

Dogs anaesthetized with pentobarbitone were used. After opening the abdomen, the ureters were cannulated with polyethylene tubing. The blood pressure was recorded from the femoral artery, and an infusion of physiological saline was given into a femoral vein at a constant rate (0.2 ml./kg./min.) throughout the experiment. Total urine samples were collected every 30 min. The urine was passed through a column of alumina, and determinations of adrenaline and noradrenaline were carried out by the fluorimetric method described by Millar and Benfey (1958).

Drugs were usually given after a control period of 60 min. (two 30 min. collections). The following agents were employed: phenoxybenzamine hydrochloride (Dibenzyline, kindly supplied by Messrs. Smith, Kline and French, Montreal), which was dissolved in a small quantity of propylene glycol, brought to a volume of 50 ml. with physiological saline and injected intravenously over a 10 min. period; (−)-adrenaline bitartrate and (−)-noradrenaline bitartrate monohydrate (kindly supplied by Sterling Winthrop Research Institute, Rensselaer, N.Y.); acetylcholine chloride (Hoffmann-LaRoche); methacholine chloride (Merck); and hexamethonium bromide (Vegolysen, kindly supplied by Messrs. Poulenc Ltd., Montreal). Amounts of adrenaline and noradrenaline given below refer to the base.

RESULTS

Tables I and II contain observations from six experiments in which 10 and 20 mg./kg. of phenoxybenzamine respectively were injected. It is evident that after phenoxybenzamine output of both adrenaline and noradrenaline in the urine was increased, and that this increase was sustained for a long time. These changes were associated with an early moderately-increased urine output and a temporary fall of blood pressure. Fig. 1 shows the mean concomitant changes in urinary adrenaline and noradrenaline, urine flow, and blood pressure in these experiments. It is clear that towards the end of each experiment, although the blood pressure level was being restored to the control level, the rate of excretion of the amines was still progressively increasing.

When the effects of phenoxybenzamine were studied similarly during a continuous infusion of hexamethonium, as shown in Table III, the output of adrenaline and noradrenaline was not

TABLE I

URINARY EXCRETION OF ADRENALINE (A) AND OF NORADRENALINE (N) BEFORE AND AFTER PHENOXYBENZAMINE

Urinary catechol amine estimations are given in ng./kg./min. No urine secreted after 7th 30 min. period in Expt. No. 2; 9th period sample not analysed in Expt. No. 3.

Expt. No.:	1		2		3		4		5		6		Mean (1 to 6)	
30 min. Period	A	N	A	N	A	N	A	N	A	N	A	N	A	N
1	0	0	0·6	0·7	1·5	1·1	0	0	0	0	0·1	0·5	0·4	0·4
2	0	0	1·1	0·2	2·4	1·5	1·3	0	0	0	0·1	0·5	0·8	0·4
	Phenoxybenzamine 10 mg./kg.						Phenoxybenzamine 20 mg./kg.							
3	0·5	2·5	3·1	0	3·1	0·8	0	0	0·5	1·2	0·1	3·4	1·2	1·3
4	2·0	5·0	3·1	0·4	2·9	0·6	2·6	7·1	2·5	1·7	0	9·4	2·2	4·0
5	2·5	4·1	4·7	0·3	4·3	1·9	3·0	3·3	6·0	2·2	0	2·6	3·4	2·4
6	2·5	5·4	2·9	0·6	5·8	2·9	3·0	5·4	3·6	1·1	0·6	4·0	3·1	3·2
7	1·1	1·6	—	—	1·9	3·0	2·0	2·6	13·6	5·1	0·6	4·0	3·8	3·3
8	1·2	2·7	—	—	1·1	2·1	1·6	2·3	17·0	9·5	11·0	5·6	6·4	4·4
9	1·2	3·1	—	—	—	—	1·0	1·0	13·6	12·5	11·0	5·6	6·7	5·5

augmented. The rate of urine secretion increased as usual, but there was no fall of blood pressure. Indeed, despite the continued infusion of hexamethonium, there was a small increase in blood pressure following the phenoxybenzamine injection. It was clear from the above that, during ganglionic block, phenoxybenzamine did not induce an increased output of adrenaline and noradrenaline, and the associated depressor response was also blocked.

In order to see if the increased excretion of the catechol amines after phenoxybenzamine resulted from a reflex response to the hypotension produced by the drug, experiments were performed in which subcutaneous injections of methacholine were given in small repeated doses in order to simulate the hypotension following phenoxybenzamine.

Table IV shows that, during methacholine injections, there was a decrease in mean blood pressure from the control readings of 147 and 139 mm. Hg (first and second period) to 108, 93, and 82 mm. Hg during the third, fourth, and fifth periods respectively. Whilst the blood pressure was depressed, the excretion of adrenaline was increased, but that of noradrenaline was not. Assuming that methacholine acts only at the end-organs innervated by the parasympathetic, these findings would suggest that the increased adrenaline output was caused by reflex release

TABLE II

MEAN BLOOD PRESSURE AND URINE SECRETION BEFORE AND AFTER PHENOXYBENZAMINE

Blood pressure (B.P.) in mm. Hg and urine secretion in ml./kg./min.

Expt. No.:	1		2		3		4		5		6		Mean (1 to 6)	
Period	B.P.	U	B.P.	U	B.P.	U	B.P.	U	B.P.	U	B.P.	U	B.P.	U
1	166	0·01	104	0·14	160	0·08	138	0·02	130	0·03	160	0 01	143	0·05
2	154	0·01	90	0·06	150	0·10	140	0·02	138	0·04	158	0·02	138	0·04
	Phenoxybenzamine 10 mg./kg.						Phenoxybenzamine 20 mg./kg.							
3	96	0·01	94	0·08	116	0·09	78	0·05	108	0·12	90	0·16	97	0·09
4	78	0·01	82	0·08	112	0·09	98	0·02	88	0·16	70	0·04	88	0·07
5	84	0·01	54	0·06	112	0·14	104	0·05	64	0·07	68	0·01	81	0·06
6	100	0·01	40	0·02	120	0·21	108	0·04	60	0·02	72	0·01	84	0·05
7	118	0·01	38	0	122	0·18	102	0·02	74	0·02	84	0·01	90	0·04
8	130	0·01	38	0	120	0·14	110	0·02	90	0·03	84	0·02	95	0·04
9	140	0·01	—	—	—	—	117	0·03	118	0 04	84	0 02	115	0·03

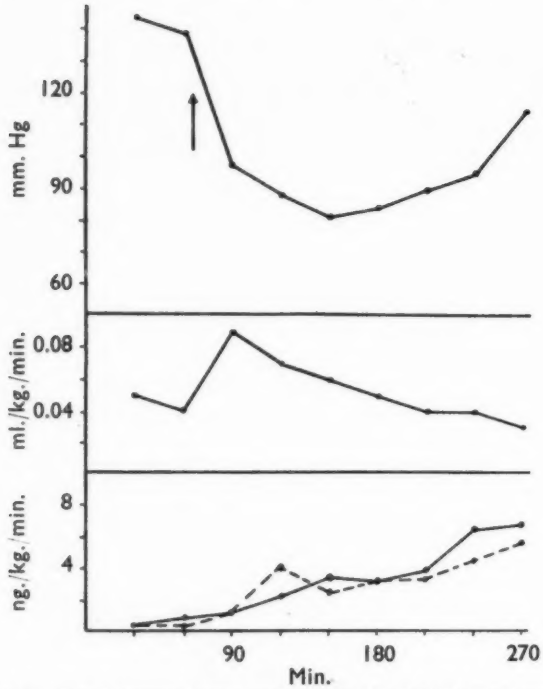

FIG. 1.—Blood pressure (upper curve), urine flow (middle curve) and urinary excretion (lowest two curves) of adrenaline (full line) and of noradrenaline (broken line) before and following administration (at arrow) of phenoxybenzamine (means of observations in Tables I and II).

of the amine from the adrenal medulla due to the fall of blood pressure. However, hypotension may not account for the equally high noradrenaline output following phenoxybenzamine.

TABLE III

MEAN URINARY EXCRETION OF ADRENALINE AND OF NORADRENALINE, RATE OF URINE SECRETION, AND BLOOD PRESSURE BEFORE AND DURING HEXAMETHONIUM INFUSION AND BEFORE AND AFTER PHENOXYBENZAMINE INJECTION

All values were based on 3 experiments. Means with range in parentheses.

30 min. Period	Adrenaline (ng./kg./min.)	Noradrenaline (ng./kg./min.)	Urine (ml./kg./min.)	B.P. (mm. Hg)
1	0·3 (0·0–0·7)	0·0 (0·0–0·1)	0·03 (0·01–0·05)	162 (146–178)
2	0·2 (0·0–0·4)	0·1 (0·0–0·1)	0·05 (0·01–0·08)	155 (132–179)
Hexamethonium (0·1 mg./kg./min.) infusion started				
3	0·4 (0·0–1·1)	0·1 (0·0–0·4)	0·05 (0·01–0·08)	103 (48–130)
4	0·0 (0·0–0·0)	0·0 (0·0–0·0)	0·03 (0·00–0·06)	91 (62–120)
Phenoxybenzamine injection (20 mg. kg.)				
5	0·3 (0·0–0·6)	0·1 (0·0–0·2)	0·06 (0·01–0·09)	105 (68–124)
6	0·0 (0·0–0·1)	0·1 (0·0–0·3)	0·07 (0·01–0·11)	97 (64–120)
7	0·4 (0·0–1·1)	0·1 (0·0–0·4)	0·07 (0·02–0·12)	106 (80–138)
Hexamethonium stopped				

TABLE IV

URINARY EXCRETION OF ADRENALINE AND OF NORADRENALINE, RATE OF URINE SECRETION AND BLOOD PRESSURE BEFORE, DURING, AND AFTER METHACHOLINE INJECTED SUBCUTANEOUSLY

All values are based on 3 experiments. Means with range in parentheses.

30 min. Period	Adrenaline (ng./kg./min.)	Noradrenaline (ng./kg./min.)	Urine (ml./kg./min.)	B.P. (mm. Hg)
1	0·2 (0·0–0·6)	0·3 (0·0–0·5)	0·11 (0·03–0·14)	147 (143–153)
2	0·6 (0·0–1·2)	0·3 (0·0–0·5)	0·16 (0·13–0·18)	139 (138–140)
Methacholine injections started				
3	0·4 (0·0–1·1)	0·3 (0·0–0·6)	0·08 (0·02–0·16)	108 (90–120)
4	1·4 (0·1–2·1)	0·2 (0·0–0·6)	0·05 (0·01–0·11)	93 (83–104)
5	1·3 (1·3)	0·1 (0·1)	0·03 (0·00–0·05)	82 (80–84)
Methacholine stopped				
6	3·6 (0·0–7·2)	0·5 (0·0–1·0)	0·02 (0·00–0·06)	105 (86–118)
7	0·9 (0·1–1·3)	0·2 (0·0–0·4)	0·04 (0·01–0·10)	122 (99–138)
8	0·1 (0·0–0·1)	0·3 (0·0–0·6)	0·03 (0·03)	113 (92–134)
9	0·1 (0·0–0·1)	0·3 (0·0–0·5)	0·05 (0·03–0·06)	119 (100–138)

In order to test the effects of phenoxybenzamine on the recovery from the urine of amines released in greater amounts within the body, 5 mg./kg. of acetylcholine was injected intravenously every 3 min. after atropine had been given. Table V shows that during two successive periods (the third and fourth) during which acetylcholine was administered before phenoxybenzamine there

TABLE V

URINARY EXCRETION OF ADRENALINE AND OF NORADRENALINE, RATE OF URINE SECRETION, AND BLOOD PRESSURE BEFORE AND AFTER PHENOXYBENZAMINE INJECTION

During periods 3, 4, 8 and 9 (marked *) atropine (2 mg./kg.) followed by 10 injections of acetylcholine (5 mg./kg.) at 3 min. intervals were given. All values are based on 4 experiments. Means with range in parentheses.

30 min. Period	Adrenaline (ng./kg./min.)	Noradrenaline (ng./kg./min.)	Urine (ml./kg./min.)	B.P. (mm. Hg)
1	0·2 (0·0–0·5)	0·7 (0·0–1·6)	0·03 (0·01–0·06)	135 (70–197)
2	0·6 (0·0–1·1)	0·4 (0·0–1·6)	0·03 (0·01–0·07)	149 (102–186)
*3	2·6 (0·1–4·6)	0·6 (0·0–1·2)	0·02 (0·01–0·03)	191 (100–270)
*4	4·8 (2·8–6·6)	0·8 (0·0–2·1)	0·02 (0·01–0·03)	175 (110–230)
Phenoxybenzamine (10 mg./kg.)				
5	1·0 (0·7–1·5)	0·4 (0·1–1·4)	0·05 (0·01–0·11)	87 (70–116)
6	0·7 (0·2–0·9)	1·1 (0·0–2·5)	0·05 (0·03–0·07)	72 (58–92)
7	0·8 (0·0–1·5)	0·9 (0·1–1·9)	0·04 (0·03–0·05)	73 (58–92)
*8	3·9 (1·4–8·3)	2·4 (0·2–4·5)	0·04 (0·01–0·08)	55 (44–69)
*9	2·7 (0·4–5·7)	2·4 (0·5–4·0)	0·04 (0·01–0·12)	51 (43–60)
10	0·4 (0·0–0·7)	0·4 (0·0–0·6)	0·07 (0·01–0·17)	94 (70–134)

TABLE VI

CHANGES IN URINARY EXCRETION OF ADRENALINE (A) AND OF NORADRENALINE (N), URINE SECRETION (U), BLOOD PRESSURE (B.P.), DURING INFUSIONS OF ADRENALINE WITH AND WITHOUT PHENOXYBENZAMINE

The infusion of adrenaline was at the rate of 0·5 μg./kg./min. Estimates of A and N are in ng./kg./min.; urinary secretion in ml./kg./min. and blood pressure in mm. Hg. All values are means of four experiments with phenoxybenzamine and three without, with ranges in parentheses.

30 Min. Period	A	N	U	B.P.	A	N	U	B.P.
1	4·8 (2·5–8·9)	0·2 (0–0·4)	0·07 (0·03–0·1)	106 (98–114)	1·1 (0·5–2·1)	1·2 (0·7–1·9)	0·22 (0·07–0·28)	138 (90–170)
2	6·5 (3·0–9·7)	0·3 (0–0·8)	0·08 (0·01–0·17)	98 (68–146)	1·4 (0·8–1·9)	1·1 (0·3–1·9)	0·27 (0·08–0·48)	131 (98–162)
	Adrenaline infusion started				Adrenaline infusion started			
3	18·7 (16·5–21·5)	0·3 (0–0·8)	0·11 (0·01–0·27)	111 (88–138)	19·3 (12·6–24·1)	0	0·21 (0·10–0·34)	135 (100–166)
4	23·5 (20·5–28·2)	0	0·10 (0·02–0·21)	111 (90–136)	23·5 (17·0–27·8)	0·9 (0–3·6)	0·18 (0·12–0·29)	144 (112–166)
					Phenoxybenzamine (10 mg./kg.) injected			
5	20·8 (16·8–24·0)	0	0·09 (0·02–0·16)	114 (98–138)	26·2 (21·1–30·0)	0·7 (0–2·6)	0·17 (0·11–0·27)	101 (90–126)
6	21·0 (17·6–25·4)	0	0·09 (0·03–0·13)	115 (100–138)	27·7 (19·2–36·6)	1·1 (0–4·2)	0·13 (0·07–0·21)	89 (70–116)
7	18·1 (17·0–19·5)	0·1 (0–0·3)	0·08 (0·03–0·13)	118 (100–146)	27·2 (17·3–39·1)	1·2 (0–4·9)	0·09 (0·04–0·16)	85 (62–106)
8	20·9 (17·3–23·8)	0	0·09 (0·04–0·13)	124 (106–146)	23·0 (15·0–33·2	1·3 (0–5·2)	0·08 (0·03–0·14)	88 (66–108)
9	18·8 (18·6–19·2)	0	0·11 (0·05–0·16)	122 (94–146)	27·7 (22·7–31·6)	2·4 (0–9·4)	0·09 (0·04–0·14)	91 (70–108)
10	21·5 (19·9–23·2)	1·3 (0–3·8)	0·12 (0·08–0·16)	119 (90–146)	23·5 (21·8–27·6)	2·7 (0–9·4)	0·10 (0·09–0·14)	88 (66–104)
	Adrenaline stopped				Adrenaline stopped			
11	9·0 (4·1–13·6)	0·5 (0–0·9)	0·11 (0·07–0·15)	109 (80–146)	11·0 (6·6–16·4)	3·5 (0·4–6·1)	0·14 (0·06–0·18)	99 (90–112)
12	3·7 (0–8·9)	0	0·10 (0·06–0·16)	104 (70–146)	3·3 (1·9–6·5)	2·8 (0·2–4·7)	0·18 (0·04–0·25)	89 (78–104)

was an increased excretion of adrenaline, but no significant change in noradrenaline. Variable pressor responses were observed after each injection of acetylcholine; the mean blood pressure during these periods reached 191 and 175 mm. Hg respectively, compared with the mean control values of 135 and 149 mm. Hg.

Following this, phenoxybenzamine induced only a slightly increased output of the amines, which was associated with an increased urine flow and a fall of blood pressure. Further acetylcholine injections after phenoxybenzamine (in the eighth and ninth periods) again increased the adrenaline excretion and, this time, increased the excretion of noradrenaline as well. These changes were associated with characteristic depressor (reversal) effects. Since identical "stimuli" were applied to release the amines before and after phenoxybenzamine, it would appear that phenoxybenzamine augmented not the excretion of endogenously-released adrenaline but that of noradrenaline.

It was of interest that, when a single injection of acetylcholine (5 mg./kg.) was given after atropine, there was always a good pressor response, but no significant change in the quantities of the amines excreted in the corresponding 30 min. sample was demonstrable. However, when adrenaline and noradrenaline were injected in amounts which had equivalent effects upon the blood pressure, excretion of the amines in the urine was greatly increased. It would appear, therefore, that either the amines released endogenously by acetylcholine are more active than those injected, or that, after release, they were so rapidly inactivated that none was detectable in the urine, except on repeated stimulation with acetylcholine. Our results permit no conclusion regarding the quantities of the amines released in this way.

With the hope of throwing some light upon the effects of phenoxybenzamine on adrenaline and noradrenaline "inactivation," we investigated the effects of the drug upon the excretion of infused adrenaline and noradrenaline. The results are shown in Tables VI and VII.

During infusion of small amounts of adrenaline (0.5 μg./kg./min.) without phenoxybenzamine, the output of adrenaline was increased, but that of noradrenaline slightly depressed. On the other hand, during a similar infusion of adrenaline with an injection of phenoxybenzamine added, the excretion of adrenaline was again increased, but this was associated with a progressively increasing excretion of noradrenaline. Indeed, even after the adrenaline infusion was stopped, the increased noradrenaline excretion continued. During infusion of noradrenaline, the output of noradrenaline was increased, but there was little change in that of adrenaline. On the other hand, during similar noradrenaline infusion with phenoxybenzamine added, the increased noradrenaline output was associated with some increase in adrenaline output (Table VII).

Assuming that the basal rate of excretion of the amines is constant and equal to that obtained in the control period, it was calculated that, during the first 3 hr. of infusion of adrenaline alone, 3% of the injected adrenaline was recovered in the urine, while in the 3 hr. period with phenoxybenzamine present the recovery was somewhat higher, 4.3%, although this difference appears unlikely to be significant. On the other hand, during noradrenaline infusion alone, 1.8% was recovered during the first 3 hr., while with phenoxybenzamine present the recovery was 1.9%. It must be concluded, therefore, that under the present experimental conditions the

TABLE VII

CHANGES IN URINARY EXCRETION OF ADRENALINE (A) AND NORADRENALINE (N), URINE SECRETION (U), BLOOD PRESSURE (B.P.), DURING INFUSIONS OF NORADRENALINE WITH AND WITHOUT PHENOXYBENZAMINE

Estimates of A and N are given in ng./kg./ml.; urinary secretion in ml./kg./min. and blood pressure in mm. Hg. The infusion of noradrenaline was at the rate of 0·5 μg./kg./min. All values are the means of four experiments with phenoxybenzamine and three without, with ranges in parentheses.

30 Min. Period	A	N	U	B.P.	A	N	U	B.P.
1	1·9 (0–2·9)	0·7 (0–2·2)	0·14 (0·04–0·29)	123 (102–148)	0·4 (0–0·6)	0·7 (0–1·9)	0·07 (0·04–0·10)	129 (121–140)
2	3·5 (0–6·1)	0·1 (0–0·4)	0·16 (0·09–0·25)	113 (98–135)	1·4 (0–3·8)	0·3 (0–1·2)	0·10 (0·05–0·18)	119 (88–140)
	Noradrenaline infusion started				Noradrenaline infusion started			
3	3·0 (0–5·7)	7·5 (4·3–13·2)	0·16 (0·10–0·22)	115 (94–141)	1·1 (0·3–1·9)	7·0 (1·6–11·2)	0·08 (0·04–0·14)	126 (100–150)
4	2·9 (0·1–6·4)	10·0 (7·7–13·5)	0·11 (0·07–0·15)	118 (94–140)	0·8 (0–1·5)	13·1 (9·1–19·0)	0·09 (0·04–0·12)	124 (92–148)
					Phenoxybenzamine (10 mg./kg.) injected			
5	2·2 (0–3·9)	10·0 (6·3–17·5)	0·11 (0·07–0·17)	125 (98–148)	0·7 (0–1·6)	11·3 (4·1–18·7)	0·08 (0·05–0·13)	91 (68–128)
6	1·3 (0·8–1·7)	9·5 (7·0–10·8)	0·13 (0·08–0·20)	133 (106–154)	3·0 (0–4·6)	12·5 (10·7–15·8)	0·04 (0–0·09)	67 (45–97)
7	1·8 (0·9–2·9)	9·6 (7·1–10·9)	0·15 (0·10–0·20)	145 (118–164)	6·1 (0–11·2)	15·7 (13·9–18·6)	0·04 (0–0·07)	70 (50–104)
8	1·6 (0·9–2·3)	10·0 (5·6–13·2)	0·14 (0·11–0·17)	141 (120–164)	3·3 (0–9·4)	13·0 (6·2–17·1)	0·06 (0·03–0·08)	77 (56–121)
9	1·7 (1·6–1·9)	8·0 (6·1–10·0)	0·13 (0·11–0·15)	126 (118–134)	2·0 (0–6·2)	12·4 (5·6–15·9)	0·05 (0·03–0·07)	66 (60–73)
10	1·5 (1·4–1·5)	7·8 (5·2–10·5)	0·14 (0·13–0·14)	117 (104–130)	2·4 (1·5–3·4)	12·7 (8·6–16·9)	0·08 (0·08)	70 (64–76)
	Noradrenaline stopped				Noradrenaline stopped			
11	0·9 (0·2–1·6)	2·1 (0·6–2·9)	0·13 (0·10–0·14)	113 (102–126)	1·0 (0–2·2)	9·2 (3·7–17·1)	0·08 (0·04–0·13)	106 (84–135)
12	0·8 (0–1·6)	1·2 (0–2·8)	0·12 (0·08–0·17)	110 (98–120)	0·5 (0·1–0·6)	2·1 (0–5·7)	0·10 (0·05–0·13)	104 (84–138)

recovery in the urine of the small amounts of adrenaline or of noradrenaline infused was not significantly changed by phenoxybenzamine.

It was observed that, during 3 hr. of adrenaline infusion following phenoxybenzamine, the animals excreted a mean of 0.28 μg./kg. of noradrenaline, while following phenoxybenzamine alone the excretion of noradrenaline was 0.56 μg./kg. in the first 3 hr. Similarly, in the 3 hr. of noradrenaline infusion after phenoxybenzamine, the animals excreted a mean of 0.52 μg./kg. of adrenaline, whereas in the first 3 hr. after phenoxybenzamine alone the total excretion of adrenaline was 0.61 μg./kg. It may be concluded then that infusions of adrenaline or noradrenaline reduced the effect of phenoxybenzamine.

This may also account for the failure of the urine catechol amine content to increase markedly following 1 hr. of ganglionic stimulation by acetylcholine (Table V) which led to an increased output of amines in the urine.

In the above experiments, the infusions of adrenaline and noradrenaline in the dose employed (0.5 μg./kg./min.) did not appreciably influence the blood pressure or the rate of urine secretion. However, after phenoxybenzamine in both groups of experiments there was the usual fall of blood pressure and a slight decrease in urine flow.

Discussion

Our results indicate that the increased urinary adrenaline excretion following the administration of phenoxybenzamine was due to nervous stimulation of the adrenal gland since this effect was blocked by hexamethonium. It may be a reflex elicited by the hypotension. Dontas and Nickerson (1957) reported an increase in the activity of the splanchnic nerve in cats following phenoxybenzamine which could be greatly reduced by tetraethylammonium. However, a reflex stimulation of the adrenal gland due to hypotension appears to account only partly for the elevated adrenaline in the urine. The methacholine hypotension caused only a moderate rise in adrenaline. Similarly, hypotension in the early stages of haemorrhage elevated the content of adrenaline only moderately (Millar and Benfey, 1958). We may, therefore, assume that part of the increased adrenaline in the urine arose from stimulation of sympathetic centres.

Our results do not afford any clue to the cause of the increased urinary noradrenaline. Brown and Gillespie (1957) and Brown, Davies, and Gillespie (1958) reported that, following phenoxybenzamine, greater amounts of noradrenaline could be recovered in the venous outflow of organs whose sympathetic supply was stimulated electrically. They suggested that inactivation of sympathetic transmitter substance occurred at receptor sites. Phenoxybenzamine interferes with the inactivation of noradrenaline by blocking sympathetic receptor sites. These authors do not appear to have investigated the effects of phenoxybenzamine on plasma noradrenaline in the absence of electrical stimulation. However, our results may be explained on their view that the increased urinary noradrenaline after phenoxybenzamine is derived from postganglionic sympathetic fibres, and is not adequately destroyed at receptor sites when these are blocked by phenoxybenzamine. The effect is not seen when hexamethonium paralyses the peripheral sympathetic system, because it depends on the state of activity of the sympathetic nervous system and is thus greatest in conditions of heightened sympathetic tone, as after ganglionic stimulation by acetylcholine or in hypotension. In the absence of phenoxybenzamine, hypotension induced by methacholine did not elevate urinary noradrenaline significantly, nor was there any significant rise in the content of noradrenaline in the early stages of haemorrhagic hypotension (Millar and Benfey, 1958). Recent studies on the plasma of normal and adrenalectomized dogs have shown that phenoxybenzamine elevated the concentration of amines, an effect which was greatly potentiated, in the case of noradrenaline, in adrenalectomized dogs subjected to acute and irreversible haemorrhage (Millar, Keener, and Benfey, 1958, 1959).

It is quite possible that the effect described by Brown and his colleagues is demonstrable only with endogenous noradrenaline. This is certainly handled differently by the body in comparison to exogenous noradrenaline (as indicated above in connexion with the effects of acetylcholine). This might explain our negative results with the infusion of small doses of adrenaline and noradrenaline before and after phenoxybenzamine. When large amounts of the amines were injected into dogs anaesthetized with phenobarbitone, a higher recovery of noradrenaline was obtained when antisympathomimetic substances were given (Benfey, Mazurkiewicz, and Melville, 1958a and b).

Our results also suggest that endogenous and exogenous adrenaline and noradrenaline

depressed ganglionic transmission as has previously been shown by Marrazzi (1939), Stehle and Melville (1943), and King and Marrazzi (1952). This effect may account for the reduction in urinary noradrenaline during adrenaline infusion, for the reduction in urinary adrenaline during noradrenaline infusion, and for the lessened effect of phenoxybenzamine when the amines were infused, as outlined above.

Whether or not a peripheral action of phenoxybenzamine on receptor sites accounts for the effect of noradrenaline cannot be stated definitely. Our findings could also suggest that phenoxybenzamine stimulates sympathetic centres and releases catechol amines, rather like reserpine. To what extent either or both of these actions is involved requires further study.

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THE EFFECT OF ALKYLATING AGENTS ON MALE RAT FERTILITY

BY

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The effects of tumour inhibitory doses of tretamine (triethylenemelamine), busulphan, and melphalan on the fertility of male rats have been examined. The aromatic nitrogen mustard, melphalan, was inactive, but busulphan has a highly selective action on spermatogenesis which contrasts strikingly with that of tretamine. The main action of tretamine was exerted upon spermatocytes or spermatids, but, with increasing dose, the effects spread to involve a wide range of spermatogenic cells including mature sperm, so that infertility could be induced very rapidly. Busulphan, however, interfered with the development of spermatogonia for several weeks, although other germinal cells were unaffected and continued to develop into mature spermatozoa. This accounted for the continuation of normal fertility for 7 weeks after a dose, before sterility suddenly developed. The antifertility activity of tretamine could be simulated by a variety of other ethyleneimino compounds, potency being greatest in trifunctional and least in monofunctional compounds. The latter were, however, very destructive to the seminiferous epithelium with increasing dose. In the rat, there appeared to be no definite relationship between the ability of alkylating substances to interfere with the activity of normal and pathological proliferating tissues, as represented by the germinal epithelium, haematopoietic, and tumour tissue. Although carcinogenicity was a biological property of alkylating agents, other chemical types of carcinogen did not interfere with fertility.

In small doses, tretamine (triethylenemelamine) produces selective effects on spermatogenesis in rats, as revealed by fertility studies (Bock and Jackson, 1957). It appears that the drug can interfere with various stages of this complex process so that the sperm produced, although capable of reaching and penetrating ova, are incapable of promoting further development. Tretamine is well known for its cytotoxic effects due, it is thought, to some specific action on dividing cells. Because of its similarity to radiation, tretamine is classified as a radiomimetic drug. The present work describes the comparative actions on male rat fertility of tretamine, busulphan (Myleran), and melphalan as representative of three chemical types of alkylating radiomimetic substances. In addition the activities of a number of tri-, di-, and monofunctional ethyleneimino compounds have been examined.

METHODS AND MATERIALS

The animals used were an inbred strain of Wistar rat of American origin, maintained on a standard diet provided by the Scottish N.E. Agricultural Society. The method of investigating the effect of a drug on male rat fertility has been described previously (Bock and Jackson, 1957). Briefly, it consisted of pairing treated male rats with females of established fertility, and replacing the latter at intervals of 1 week for as long as considered necessary. The date of insemination was determined by examination of a vaginal smear each morning. Because of the number of females required in each experiment, the testing of individual substances has been limited to one or two concentrations related, where possible, to existing information concerning relevant biological activity, such as tumour inhibition, carcinogenicity or interference with the haemopoietic system. Otherwise, the total dose used was close to the maximum tolerated. The majority of substances were administered daily by the intraperitoneal route for 5 days and pairing commenced the first day of the next week, namely day 8 from the first dose. This permitted at least 72 hr. for surplus drug to be eliminated. During the third week after mating, the females were inspected for pregnancy in case litters should be destroyed at birth. The administration of short courses of treatment, rather than single doses, made it less likely that antifertility activity would be missed.

Since ethyleneimino compounds have the reputation of instability, a careful check was maintained on these substances. Only with diethyleneurea was there any reason to suspect deterioration of the pure compound with time. As this was manifest by enhanced toxicity, the compound was freshly prepared before use. All the compounds were kept in tightly stoppered containers at −20° and allowed to warm up to room temperature before opening to avoid condensation of water on to the contents.

Solutions for injection were prepared immediately before use, mainly in water, although arachis oil had to be used in some instances. Nitrofurantoin (Furadantin) was injected as a suspension in 0.5% sodium carboxymethylcellulose solution, whilst nitrofurazone (Furacin) was administered to male rats in the diet as a paste. This latter procedure necessitated an adaptation of the males and females to feeding separately during the day only, with introduction of the female to the male each evening.

Generally speaking, the doses used did not depress the sexual activity of the males so that the great majority of females were inseminated during the series of pairings. The average litter size was calculated by dividing the total viable offspring by the number of females known to have been inseminated. There was always a close correspondence between visible pregnancies and the production of litters, a reassurance that the drugs caused no increased tendency for litter destruction due to abnormal offspring.

Results

The formulae of the important substances referred to in Tables I, II and III are shown in Fig. 1. The terms tri-, di-, and mono-functional simply refer to the number of chemically reactive groups (alkylating groups) present in the molecule.

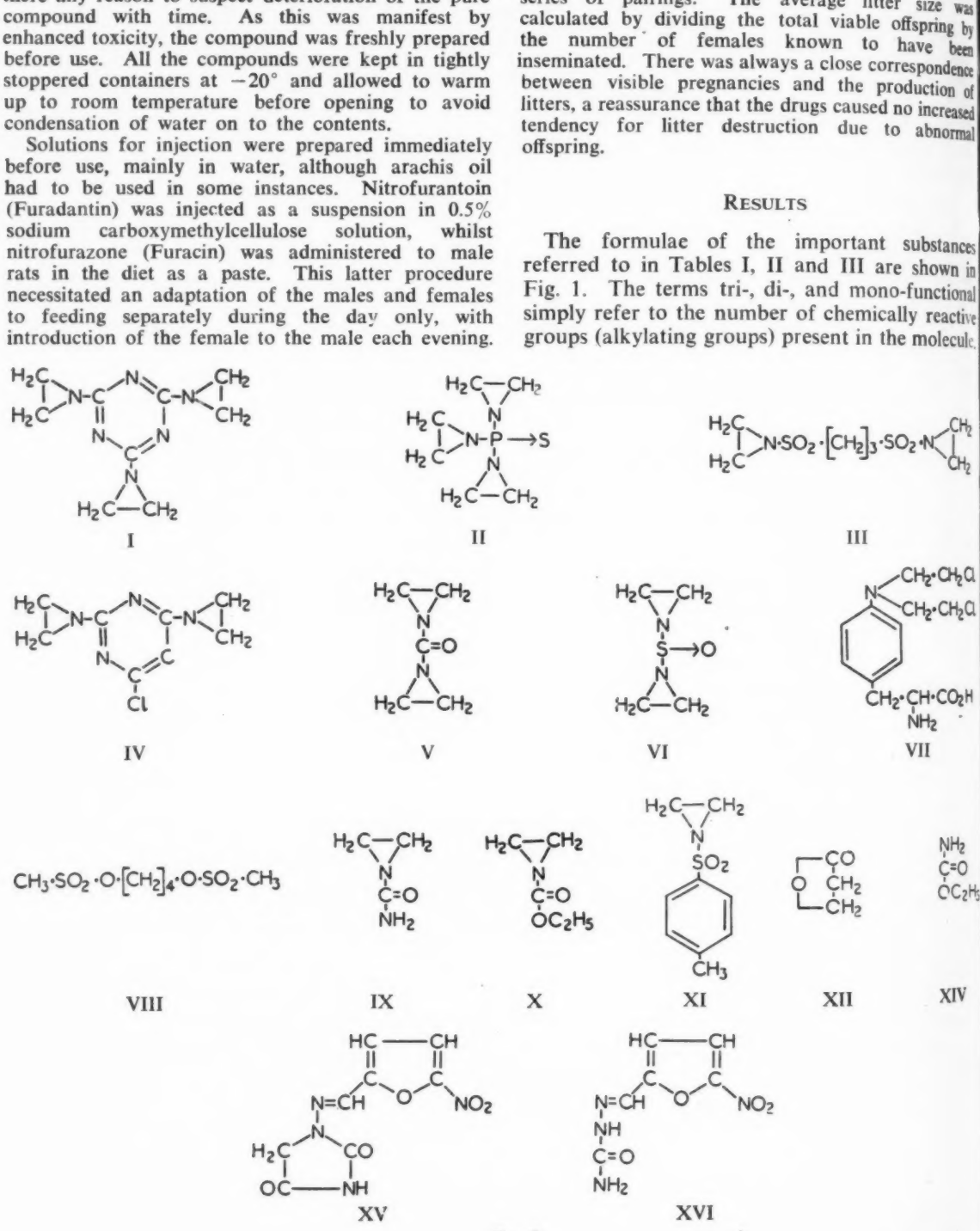


FIG. 1

Tri- and Di-functional Ethyleneimino Compounds

The results with these compounds are recorded in Table I.

Tretamine (I).—After five daily doses of 0.2 mg./kg., the animals were sterile until the sixth week after the first dose. Between the 37th and 46th day fertility returned, the average litter size being normal by the 8th week. The effect of one dose (0.2 mg./kg.) was infertility 4 weeks later (Bock and Jackson, 1957).

Thiotepa (triethylenethiophosphoramide) (II).—Five daily doses of 0.4 mg./kg. produced a similar effect to that of tretamine except that sterility was not quite complete in week 2. Inseminations from the 37th day onwards resulted in normal size litters.

1,3-Di(ethylenesulphamoyl)propane (III).—1,3-Di(ethylenesulphamoyl)propane (see Paterson and Kunkler, 1954) in five doses of 1.8 mg./kg. induced sterility in most males for four weeks after treatment with return to normal fertility by the 6th week.

6-Chloro-2,4-diethyleneiminopyrimidine (IV).—The quantity of this compound used was well below that required to inhibit the growth of the Walker tumour (Hendry, Homer, Rose, and Walpole, 1951). After a total dose of 1 mg./kg. over 5 days, the animals remained practically sterile for 3 weeks with return to normal fertility by the 6th week. The pattern of events was similar to that following treatment with tretamine and thiotepa.

Diethyleneurea (V).—10 mg./kg. over 5 days only produced a lower average litter size during weeks 2 to 5. The dosage used, which was close to that causing lethal effects, also effectively inhibited the growth of the Walker tumour.

TABLE I

EFFECT OF TRI- AND DI-FUNCTIONAL ALKYLATING SUBSTANCES ON THE FERTILITY OF MALE RATS

Each treated male was paired in consecutive weeks with a different female. Since males and females were of established fertility, insemination should result in litter production. The number of litters produced each week compared with the number of females known to have been inseminated is shown as a ratio in the upper line of each experiment (under mating periods) and the average litter size, representing the number of offspring/female inseminated, shown by the numerals in the lower line. Unless otherwise stated, all doses were given by the intraperitoneal route. Under treatment aq denotes aqueous solution. An asterisk denotes that doses were separated by an interval of 12 days. D denotes all rats dead by week 5.

Drug	Treatment	Mating Periods (in Weeks from First Dose)											
		1	2	3	4	5	6	7	8	9	10	11	12
I Tretamine	1×0·2 mg./kg., aq.	6/9 4·0	7/8 2·5	8/9 3·0	0/9 0·0	9/9 7·1	8/9 7·0						
	5×0·2 ,, ,, Experiment (1)	— —	0/5 0	0/5 0	0/5 0	0/5 0	2/5 1·6	5/5 6·2	4/5 7·8				
	5×0·2 mg./kg., aq. Experiment (2)	— —	0/6 0	0/6 0	0/6 0	0/6 0	2/6 1·3	5/6 5·3	5/6 8·7				
II Thiotepa	5×0·4 mg./kg., aq.	— —	3/4 2·7	0/4 0	0/3 0	0/4 0	3/5 6·0	5/5 8·2	5/5 11	4/4 11			
	5×0·4 ,, ,,	— —	— —	— —	0/5 0	3/5 0·8	5/5 9·5	4/4 10	5/5 5·6	5/5 9·6			
III 1,3-Di(ethylenesulphamoyl)propane	5×1·8 ,, in oil	— —	1/5 0·2	1/5 0·2	2/5 0·6	2/5 1·8	4/4 7·5						
IV 6-Chloro-2,4-diethyleneiminopyrimidine	5×0·2 ,, aq.	— —	0/4 0	2/6 1·5	0/8 0	3/5 2·4	7/7 8·1						
V Diethyleneurea ..	5×2 ,, ,,	— —	3/4 5·0	3/4 2·4	4/5 4·4	4/5 6·8	3/3 7·7	4/4 7·0					
VI Diethyleneiminosulphoxide	5×2·5 ,, ,,	— —	7/7 5·1	7/7 5·0	4/6 1·3	1/5 0·8	5/7 8·2	7/7 9·8					
VII Melphalan	5×0·8 ,, in oil	— —	4/5 6·8	5/5 11	4/5 8·0	5/5 7·8	5/5 10	5/5 8·6					
VIII Busulphan	1×10 ,, ,,	— —	5/5 6·8	5/5 8·8	5/5 11	4/4 8·6	5/5 9·4	5/5 6·6					
	1×10 ,, ,,	— —	— —	— —	5/5 9·8	5/5 8·4	5/5 11	5/5 11	1/5 2·0	1/5 2·4	0/4 0	0/5 0	0/5 0
	*2×10 ,, ,,	— —	— —	3/4 5·7	4/4 7·2	3/4 9·0	3/4 8·0	2/3 7·3	0/3 0	0/2 0	0/2 0		
	4×50 ,, ,, p.o.	— —	5/5 5·6	3/3 8·2	1/1 11	} D							

Diethyleneiminosulphoxide (VI).—Five daily doses of 2.5 mg./kg. produced results rather similar to those following treatment with monoethyleneurea (IX, five doses of 5 mg./kg.), except that minimal fertility occurred in the 5th instead of the 4th week. This dose approached the maximum tolerated and did not inhibit the growth of the Walker tumour.

Monofunctional Derivatives of Ethyleneimine

The results with these compounds are given in Table II.

Monoethyleneurea (IX).—Although 1 mg./kg. daily for 6 weeks had no significant effect on fertility, 5 daily doses of 5 mg./kg. produced similar changes to 1 dose of tretamine (Table I). There was a rapid decline in fertility, with sterility in the 4th week from inseminations between 22 and 26 days from the first dose. Normal fertility returned sharply in the 5th week. In contrast to the experiment with a single dose of tretamine, the 5-day course of treatment with monoethyleneurea necessitated postponement of matings until the second week of the experiment. Nevertheless infertility still occurred in all animals in the 4th week from the first dose, as after one dose of tretamine. The effect of this treatment was much more marked in hemi-castrated animals, and in these resembled the action of the short course of tretamine (5 doses of 0.2 mg./kg.). The average litter size from a corresponding group of untreated hemi-castrated males remained normal.

A higher dose rate of monoethyleneurea (5 daily doses of 10 mg./kg.) obliterated fertility for at least 8 weeks. Copulations became nearly aspermic after 7 weeks. Three animals ultimately recovered the ability to father litters of normal size. Histological examination of the testes after treatment were consistent with these findings (Fig. 2).

N-*Ethoxycarbonylethyleneimine* (X). — This ethyleneimino derivative of urethane (ethyl ethylenecarbamate) (5 daily doses of 8 mg./kg.) readily produced complete destruction of the seminiferous epithelium without apparently interfering with sexual behaviour. The animals survived for 12 months, when the experiment was terminated. None had recovered their fertility by this time, a finding consistent with the histological appearance of the testes examined 12 months after treatment. These organs showed gross damage and the epididymis remained quite empty. Inseminations from the 7th week onwards were aspermic (Table II). A smaller dose rate (5 daily doses of 4 mg./kg.) was much less effective, although producing minimal fertility in the 4th week followed by normal fertility from the 5th week. These results demonstrate a very steep dose/response effect with both monofunctional compounds IX and X.

N-*Toluene*-p-*sulphonylethyleneimine* (XI).—With a maximum tolerated dose no sterility occurred during a 3-week period following treatment.

Other Alkylating Agents

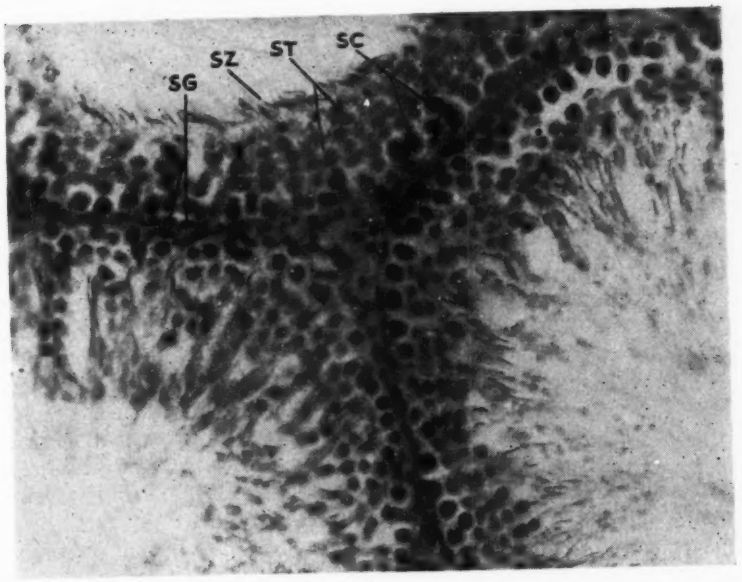
Melphalan (VII).—This difunctional nitrogen mustard derivative of L-phenylalanine had no

TABLE II

MONOFUNCTIONAL ALKYLATING SUBSTANCES AND MALE RAT FERTILITY

Each treated male was paired during consecutive weeks with a different female. Since all animals were of established fertility, litter production could be expected from each pairing. See Table I for explanation of ratio and numerals under mating periods. All doses were given by the intraperitoneal route. An asterisk indicates hemi-castrated rats.

Drug	Treatment	Mating Periods (in Weeks from First Dose)											
		2	3	4	5	6	7	8	9	10	11	12	13
IX Monoethyleneurea ..	5×5 mg./kg., aq.	5/5 6·8	5/5 3·2	0/5 0	5/5 7·0	4/4 9·4	5/5 11						
	*5×5 ,, ,,	5/6 4·7	0/5 0	0/6 0	0/6 0	5/6 6·5	6/6 8·0	4/5 8·0	5/6 7·4				
	5×10 ,, ,,	0/5 0	0/5 0	0/5 0	0/4 0	0/5 0	0/5 0	1/4 0·8	— —	— —	0/5 0	— —	1/3 2·4
	1 ,, ,, daily	5/5 12	5/5 8·6	5/5 7·4	4/4 6·2	5/5 7·8							
X *N*-Ethoxycarbonyl-ethyleneimine	5×4 ,, ,,	4/5 4·8	3/3 4·2	2/4 1·8	3/4 6·7	3/3 9·7	4/4 9·2						
	5×8 ,, in oil	1/4 0·5	0/4 0	0/4 0	0/4 0	0/3 0	0/1 0	Aspermic inseminations No further litters					
XI *N*-Toluene-*p*-sulphonyl-ethyleneimine	5×25 ,, ,,	5/5 11	5/5 8·2	4/5 9·7									



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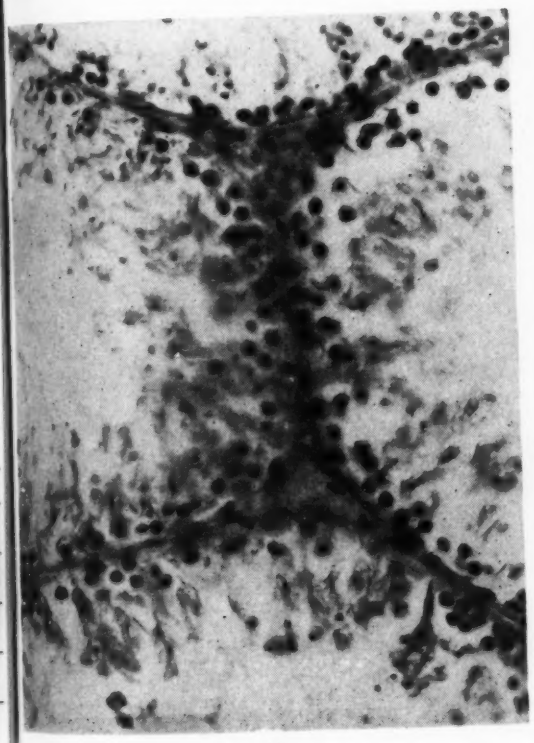

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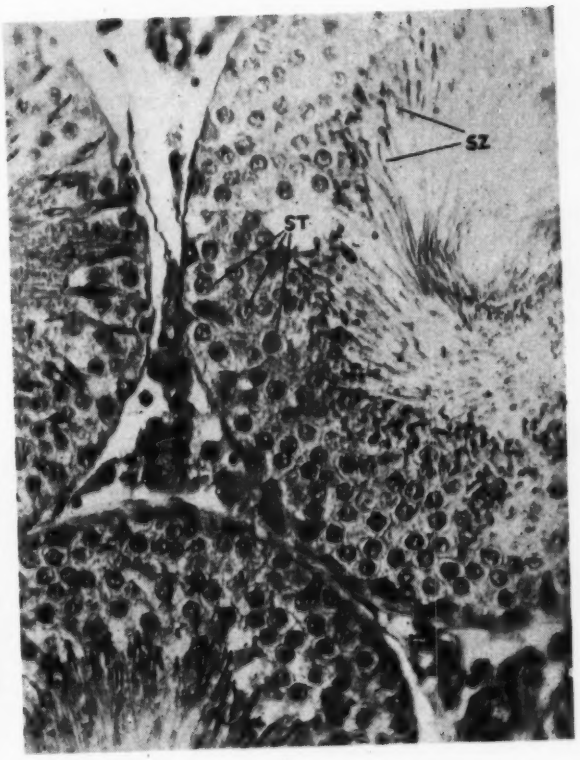

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Fig. 2.—Comparative effects at 30 days of ethyleneurea and busulphan on the testis. (*a*) Normal histological appearance of germinal epithelium after 5 daily doses of ethyleneurea (5 mg./kg., intraperitoneally). (*b*) Destructive effects of 5 daily doses of ethyleneurea (10 mg./kg., intraperitoneally). Spermatogonia and spermatocytes are no longer present. The cells are spermatids which are transforming into spermatozoa. (*c*) Germinal epithelium after 1 dose of busulphan (10 mg./kg., intraperitoneally). **SG**, spermatogonia; **SC**, spermatocytes; **ST**, spermatids; **SZ**, spermatozoa.

effect on fertility (Table I). The course of treatment given was well in excess of that required to inhibit experimental tumour growth (Bergel and Stock, 1953).

Busulphan (VIII).—A single dose (10 mg./kg.) did not interfere with fertility for 7 weeks (Table I). In this preliminary experiment, the possible later development of infertility was not suspected. In a second experiment at this dose, a delay of 3 weeks after the injection was permitted before commencing the series of pairings, and infertility appeared suddenly during week 8 in four of five animals. The remaining animal produced normal litters for 2 weeks longer before becoming abruptly sterile in the 10th week. A second dose of 10 mg./kg. 12 days after the first dose did not hasten the onset of sterility or cause subfertility, but merely enhanced the delayed lethal effects of the drug. Two of three survivors from the 2-dose experiment ultimately recovered fertility. It has again been established that delay in mating does not significantly modify the time of onset of sterility. The latter was associated with oligospermic or aspermic inseminations, indicating a sudden fall in sperm output. Even large and lethal doses such as 4 daily doses of 50 mg./kg. did not interfere with fertility up to the time of the death of the animals. It was also found that one dose of 4 mg./kg. had no effect on fertility over a period of 3 months.

β-Propiolactone (XII).—This monofunctional compound is an alkylating agent which is an effective mutagen and carcinogen (Walpole, Roberts, Rose, Hendry, and Homer, 1954). There was no evidence of antifertility action during the 6 weeks following treatment with a maximum tolerated dose (Table III).

Miscellaneous Compounds

These results are summarized in Table III.

Dibenzanthracene (XIII).—This carcinogen had no effect on fertility at a dose level which subsequently produced hepatic tumours in 4 of 5 animals.

Urethane (XIV).—This substance is a mitotic poison used in the treatment of chronic leukaemia. The dose used in the present experiments (5 daily doses of 250 mg./kg.) produced hypnosis but was not the maximum tolerated quantity; it failed to depress fertility.

Nitrofurane Derivatives (XV and XVI).—These were included because they are known to interfere with spermatocytic transformations in the testes (Nelson and Steinberger, 1953; Steinberger and Nelson, 1957). Neither compound had any significant effect on fertility during the 7 week period of the experiment.

The inactivity of compounds XII to XVI suggests that interference with spermatogenesis and fertility does not readily occur, and emphasizes that the changes in fertility after alkylating agents are not simply manifestations of general toxicity.

Discussion

Various types of alkylating compounds are known whose effects resemble those produced by radiation, so that certain normal cell systems are easily damaged by them. Whereas radiation damage is inflicted at or close to the onset of cell division, alkylating chemicals appear to act earlier, during the "resting" phase, when in fact intense synthetic activity is in progress preparatory to cell division. The result may be cell death, temporary inhibition of mitosis or the production of chromosome damage which may be manifest

Table III

MISCELLANEOUS COMPOUNDS AND MALE RAT FERTILITY

Each treated male was paired during consecutive weeks with a different female. Since all animals were of established fertility, litter production could be expected from each pairing. See Table I for explanation of ratio and numerals under mating period. All doses by the intraperitoneal route.

Drug	Treatment	Mating Periods (in Weeks from First Dose)					
		2	3	4	5	6	7
XII β-Propiolactone ..	5×20 mg.kg., in oil	4/4 5·7	5/5 8·2	5/5 9·4	5/5 7·6	3/4 7·7	
XIII Dibenzanthracene ..	4×20 ,, ,, ,,	4/4 6·5	3/4 4·2	4/4 9·5	3/3 7·7	5/5 9·4	
XIV Urethane	5×250 ,, aq.	5/5 10	5/5 9·2	5/5 8·4	5/5 8·8	4/5 6·8	
XV Nitrofurantoin ..	5×50 ,, in oil	3/3 6·3	5/5 8·2	5/5 10	4/4 8·0	5/5 9·4	5/5 8·0
XVI Nitrofurazone	0·4 mg./kg., of diet	2/2 7·5	2/2 9·5	4/4 7·5	5/6 5·7	5/5 9·6	6/6 8·3

later in the form of mutagenic or carcinogenic change. In animals, exposure to radiation or radiomimetic compounds is followed by the rapid production of leucopenia, due to damage to bone marrow and lymphoid tissue; later there may be thrombocytopenia and anaemia. These phenomena have been extensively studied, but the nature of the fundamental mechanisms involved remains unsolved.

The antifertility effects of radiomimetic chemicals have not been examined in any detail. Thus Goldeck and Hagenah (1951) described the effects of an aliphatic nitrogen mustard on spermatogenesis and fertility in the laboratory rat. Most males were sterile after injection of the maximum tolerated dose (1 mg./kg., intravenously) owing to inhibition of spermatogenesis and testicular atrophy. In some animals regeneration occurred and potency returned after 2 to 4 months. Landing, Goldin and Noe (1949) had earlier described the testicular lesions in mice following intraperitoneal injection of a number of aliphatic nitrogen mustards. They found "acute and chronic degenerative changes similar to those following metabolic deficiency, irradiation and many other chemical agents." Recovery of spermatogenic function was observed 3 to 4 weeks after non-fatal single or repeated doses. Bollag (1953) reported that busulphan, another type of alkylating radiomimetic agent, produced destructive effects on the rat testis; his results will be referred to later. Apart from the obvious question of the relation between chemical structure and ability to interfere with fertility, the major problem is to correlate changes in fertility with damage to particular types of spermatogenic cell. Our previous work (Bock and Jackson, 1957) with the ethyleneimino compound tretamine has shown that well-defined effects on fertility can be revealed by a systematic mating procedure. A brief account of more recent work with this substance, busulphan and radiation has also appeared (Craig, Fox, and Jackson, 1958).

Comparative Effects of Tretamine, Melphalan, and Busulphan

These three compounds are used in the chemotherapy of malignant disease, and represent three chemical types of alkylating agent—ethyleneimines, nitrogen mustards and sulphonyloxyalkanes respectively. The relevant features of the action of tretamine are as follows: (*a*) Sterility from inseminations occurring on 22 to 26 days from treatment, followed by recovery of normal fertility in the 5th week. (*b*) With increasing dose (Table I), a spread of drug action to involve later and earlier stages of spermatogenesis, and even to render mature sperm in the epididymis infertile. Recovery of fertility occurred 5 to 6 weeks from treatment. (*c*) The absence of noticeable oligospermia in these experiments and the retention of sperm motility and their ability to reach and penetrate ova. The onset of tretamine-induced sterility can be rapid, and the effect of this drug is cumulative so that very small doses can produce and maintain an infertility which is reversible. The 4th week sterility is probably due to interference with spermatocytes or damage to early spermatids (Craig, Fox, and Jackson, 1958; Bateman and Jackson, 1957) and not to spermatogonial damage as was suggested earlier (Bock and Jackson, 1957). Its selective nature is emphasized by the recovery of normal fertility a few days later. Similar results have since been obtained with tretamine in mice (Cattanach and Edwards, 1958) although the time relationships of the sterile periods are different from the rat.

The destructive effects of aliphatic nitrogen mustards on the germinal epithelium have already been mentioned, and in view of this the failure of the aromatic nitrogen mustard, melphalan, to impair fertility was unexpected. This shows, however, that the sensitive germinal cells in the testis may remain unharmed although marked suppression of proliferating cell systems occurs elsewhere. On the other hand, the action of busulphan has proved to be quite remarkable. According to Bollag (1953), two doses of this drug (11 mg./kg., intraperitoneally), separated by an interval of 14 days, caused extensive damage with complete destruction of the germinal epithelium in male rats examined over a period of 150 days. After one dose, the histology of the testis was said to remain normal over an interval of 30 days from the dose. In our experiments with this substance, fertility remained normal until the 8th week when sterility rapidly developed with associated oligo- or a-spermia. This clearly indicated a blocking or destructive action of the drug on an early stage of spermatogenesis. Our examination of the histology of the testis after one dose of busulphan showed that the drug interfered with spermatogenesis in a highly specific manner. The development of new spermatogonia was prevented for several weeks, although the germinal cells present at the time of treatment continued to develop into mature sperm over a period of about 45 days. In this way, the seminiferous epithelium became systematically depleted of spermatogonia, spermatocytes, spermatids and finally spermatozoa in this sequence (Fig. 2). These results account for the

continuation of normal fertility after treatment until the supply of spermatozoa is exhausted. Then follows a period of complete sterility until repopulation of the testis tubules has occurred, already apparent by the presence of numerous new spermatogonia at 45 days, and new sperm have developed and traversed the epididymis.

It is curious that increased doses of busulphan, a nucleotoxic alkylating tumour inhibitor (Haddow and Timmis, 1953), do not result in a spread of effect to adjacent spermatogenic cells, the spermatocytes, which are actively proliferating. This specificity is in striking contrast to the actions of tretamine and other ethyleneimines, and is inconsistent with a general nucleotoxic action. The progressive disappearance of germinal elements is reminiscent of that following irradiation with 500 r (Shaver, 1953 ; Steinberger and Nelson, 1957), which is due to the rapid destruction of spermatogonia (Oakberg, 1955, 1957). A suitable dose of radiation (300 r, whole body) produces a fertility pattern indistinguishable from that of busulphan (Craig, Fox, and Jackson, 1959).

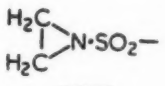
Tretamine, melphalan and busulphan inhibit the growth of a standard test tumour, the Walker carcinoma. Tretamine produces an effect on the peripheral blood which closely resembles that of both aliphatic and aromatic nitrogen mustards, namely an initial lymphocytopenia and neutropenia, followed by a marked neutrophilia (Elson, 1955, 1957). Busulphan, on the other hand, induces a selective and severe neutropenia which persists for a long time compared with the transient mustard effects. Radiation produces a general leucopenia which may be closely imitated by combined treatment with a nitrogen mustard and busulphan (Elson, 1955). It is difficult to reconcile the effects of these 3 types of alkylating radiomimetic chemicals with a common focus of attack on cellular processes.

Ethyleneimino Compounds and Fertility

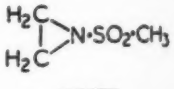
The activity of tretamine has naturally led to studies of other ethyleneimino compounds. The pharmacological activity of these substances is, in general, related to the number of ethyleneimino groups present as well as to the nature of the molecule which carries them. The results show that qualitatively similar effects on rat fertility can be produced by tri-, di- and mono-functional compounds ; their potency diminishes in this order. It is strange that spermatogonia, the most radiosensitive cells of the germinal epithelium, should be relatively insusceptible to radiomimetic compounds like tretamine, thiotepa, and 1,3-di(ethylenesulphamoyl)propane whereas later phases of spermatogenesis are very sensitive to interference by these substances.

The importance of the carrier moiety of the molecule bearing the ethyleneimino groups is seen by reference to the difunctional compounds (Table I). Diethyleneurea was ineffective even at a near lethal dose. Diethyleneiminosulphoxide caused subfertility and a short period of sterility in the fifth week after treatment but only at a dose close to the maximum tolerated quantity, whilst 6-chloro-2,4-diethyleneiminopyrimidine was highly effective at low dosage.

It was not anticipated that the monofunctional ethyleneimines would have any effect on fertility. However, both monoethyleneurea and the monofunctional urethane derivative, *N*-ethoxycarbonylethyleneimine, at intermediate dose levels caused transient sterility in the 4th week after treatment. Destruction of the germinal epithelium occurred with higher dosage with no recovery of fertility in a period of 12 months following treatment with the urethane derivative ; animals were sterile for at least 8 weeks after treatment with monoethyleneurea. Repeated doses of the latter compound (1 mg./kg. daily) failed to produce infertility whereas small daily doses of tretamine (0.05 mg./kg. daily) were cumulative (Bock and Jackson, 1957). The other monofunctional ethyleneimino compound examined, *N*-toluene-*p*-sulphonylethyleneimine, was ineffective in spite of the chemical reactivity of the alkylating group. This failure was not due to the ethyleneimino group being in a substituted sulphamoyl group



XVII



XVIII

(XVII) since *N*-methanesulphonylethyleneimine (XVIII) and 1,3-di(ethylenesulphamoyl)propane (III) were active and contain the same grouping. Comparative studies of the effects of these compounds at similar dosages are being made on the peripheral blood count of rats. The male reproductive system appears to be more susceptible to damage by ethyleneimino compounds than haemopoietic tissues. For example, monoethyleneurea induced changes in fertility at doses causing no significant alteration in the peripheral blood count. Even destruction of the germinal epithelium by this substance was associated with no more than a brief period of leucopenia.

It is interesting to compare antifertility effects with antitumour activity. With the exception of

diethyleneiminosulphoxide, all the di- and trifunctional ethyleneimino compounds examined inhibited the growth of the Walker tumour, but none of the monofunctional compounds discussed were active in this respect. Diethyleneurea, which had no antifertility effect, was a tumour inhibitor. This evidence of a dissociation of antifertility activity, haematological effects and tumour inhibition suggests that it may be possible to develop compounds with greater specificity for different proliferating cell systems.

Antifertility Activity and Carcinogenicity

A variety of monofunctional ethyleneimino compounds have been shown to be carcinogenic, but of the ethyleneimines used in the present work only tretamine is a known carcinogen in the rat, although not in aqueous solution (Walpole *et al.*, 1954; Bock and Jackson, 1957). Busulphan has induced tumours in rats (Koller, 1953) but not in mice (Shimkin, 1954; Roe and Salaman, 1955; Roe, 1957). The possibility that carcinogenicity and antifertility activity might be associated has been tested with other known carcinogens such as β-propiolactone, dibenzanthracene and urethane. None of these produced changes in fertility, so that there appears to be no consistent connection between carcinogenicity and antifertility activity.

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ASSAY OF SUXAMETHONIUM AND LAUDEXIUM ON THE FROG RECTUS ABDOMINIS

BY

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Suxamethonium, like acetylcholine, causes contracture of the isolated rectus abdominis muscle of the frog, whereas laudexium antagonizes this effect. These actions may be used to assay both drugs with reasonable speed and fiducial limits, provided that changes in responsiveness of the preparation during the course of assay are met by suitable design and analysis. Small quantities of suxamethonium and laudexium may be assayed in certain body fluids.

As chemical methods of estimating suxamethonium and laudexium are not specific or sensitive enough to study their keeping properties or *in vivo* metabolism, we have sought suitable methods of bioassay. We considered that *in vitro* methods would be preferable to *in vivo*, for reasons of speed, economy of material, precision and Home Office regulations. The isolated rat phrenic nerve diaphragm method of Mogey, Trevan, and Young (1949) cannot be employed as the rat is particularly insensitive to laudexium (Collier and Macauley, 1952). This led us to explore the use of the frog rectus abdominis preparation, with which Norton and de Beer (1954) had estimated suxamethonium, and de Jalon (1947) and Mahfouz (1949) tubocurarine.

The method of Norton and de Beer (1954), using *Rana pipiens*, entailed a recovery time of 60 to 90 min. between successive doses to the same preparation. Using *Rana temporaria*, we have developed a general method for the assay of suxamethonium, with a recovery period of 4.5 to 5 min. This method is described below, together with its extension to the assay of laudexium.

METHODS

Preparation.—All experiments were performed with *Rana temporaria*, of British or Continental origin. Continental frogs were rather more sensitive to acetylcholine and recovered more readily from laudexium. The rectus abdominis muscle was suspended in a 10 ml. organ bath at room temperature in an oxygenated Ringer solution of the following composition: NaCl 7.0 g.; KCl 0.14 g.; $CaCl_2$ (anhydrous) 0.12 g.; $NaHCO_3$ 0.2 g; glass distilled water to 1 l.

The movements of the muscle were recorded on smoked paper by an isotonic lever with a frontal writing point. This lever, which magnified the muscular movement about tenfold, applied a load of 2 to 3 g. to the muscle.

Chemicals.—Specimens of suxamethonium chloride and laudexium methylsulphate were set aside as reference standards. The chlorides of choline, succinylmonocholine, suxamethonium, acetylcholine and tubocurarine, and the methylsulphate of laudexium were used throughout, and weights are given in terms of base. These drugs were dissolved in Ringer solution and doses given in volumes not exceeding 0.5 ml. Doses of drugs added to the organ bath are expressed as weights in 10 ml.

Estimation in Body Fluids.—In estimating suxamethonium in rabbit blood, eserine sulphate was added both to the blood and to the standard suxamethonium in Ringer solution to give a concentration of 10 μg./ml. Laudexium was extracted from human urine by the method that Mahfouz (1949) used for tubocurarine. The urine was evaporated to dryness, the residue extracted with absolute ethanol, the alcoholic extract then evaporated to dryness and the residue taken up in Ringer solution.

Statistical Methods.—Analysis of variance and covariance and assessment of potencies with fiducial limits were carried out by standard statistical methods for parallel line assays. Fiducial limits corresponded to $P=0.95$.

RESULTS

Suxamethonium

Action of Drug.—Choline, succinylmonocholine and suxamethonium stimulate the frog rectus muscle (Fig. 1). The potency of suxamethonium was about 500 times that of choline and about 100 times that of the monocholine.

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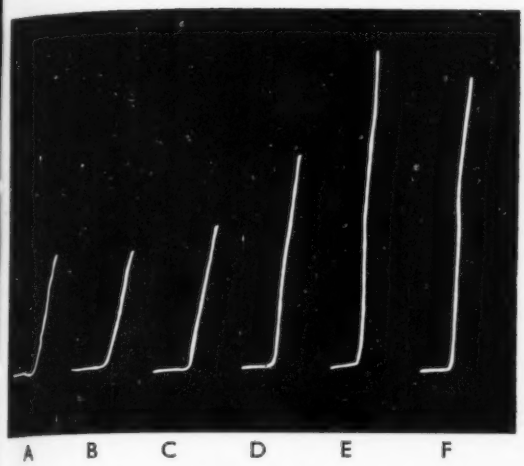

FIG. 1.—Frog rectus abdominis muscle in a 10 ml. bath. Responses to 1.5 min. of exposure to choline, succinylmonocholine, and suxamethonium. At **A**, 5 mg. and **D**, 7.5 mg. choline; at **B**, 1 mg. and **E**, 1.5 mg. succinylmonocholine; at **C**, 10 μg. and **F**, 15 μg. suxamethonium.

Dose Cycle.—The curve illustrated in Fig. 2 was obtained with exposures of the rectus muscle to suxamethonium for 2 min. followed by recovery in Ringer solution for 5 min. A dose cycle of 6 min. (1.5 min. exposure and 4.5 min. recovery) was sufficient for most preparations, and was used on many occasions.

Dose/Response Curve.—When a series of doses of different magnitude was given and the resulting contractures plotted as ordinates against log dose, a typical sigmoid curve was obtained (Fig. 2).

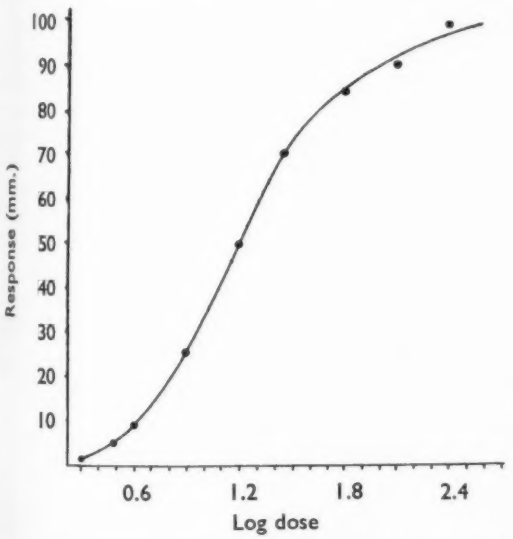

FIG. 2.—Dose response curve for suxamethonium using the frog rectus abdominis muscle. Abscissa, log dose in μg. of suxamethonium in a 10 ml. bath.

The central portion of the curve, between about 25% and 75% maximal response, was rectilinear and thus provided a basis for linear regression analysis.

Response Fall-off.—When dose cycles of 6 or 7 min. were used, the response of the preparation fell off with repeated doses. In the typical experiment illustrated in Fig. 3, sixteen doses, each of 15 μg. of suxamethonium, were given in a 6 min. cycle. The sixteenth response was 49% of the first, and regression analysis showed that the fall-off was practically rectilinear. After the 16 doses had been given the recovery period was extended to 28.5 min., making a 30 min. cycle, and 3 further doses were administered. The nineteenth response was 81% of the first.

The question arose whether the response to one dose was affected by the magnitude of the preceding response. To answer this, we performed the experiment illustrated in Fig. 4, in which alternate pairs of low and high doses of suxamethonium were given. Regression analysis showed that, within each dose group, the plot of response against time yielded a rectilinear fall-off over the assay period, although the slope of the regression line through the high doses was the steeper indicating that the true nature of the fall-off was probably exponential. Analysis also showed that the regression line through the first responses of each pair in a given dose group was parallel to that through the second responses of each pair in the same group. This showed that the size of one response did not affect that of the next.

A second experiment using alternate pairs of doses consisting of 10.0 μg. and 5.0 μg. of suxamethonium yielded similar results. These findings will be discussed in greater detail later.

Assay Design and Analysis.—Using either 6 or 7 min. dose cycles, we adopted a (2+2) pattern of assay, in which high and low doses of test and standard preparations were given in orders based on a 4×4 randomized block, a 4×4 Latin square or a 25 dose serially balanced design (Finney and Outhwaite, 1956).

The protocol of a typical assay based on a Latin square is given in Table I. The results of this assay and of assays of similar solutions of suxamethonium on randomized block and serially balanced designs are summarized in Table II.

Check on Method.—Assay of a solution containing 50 mg./ml. suxamethonium in Ringer solution gave a value of 52.30 mg./ml. with fiducial limits of 49.27 to 55.54 mg./ml. (94.2 to 106.2%). A

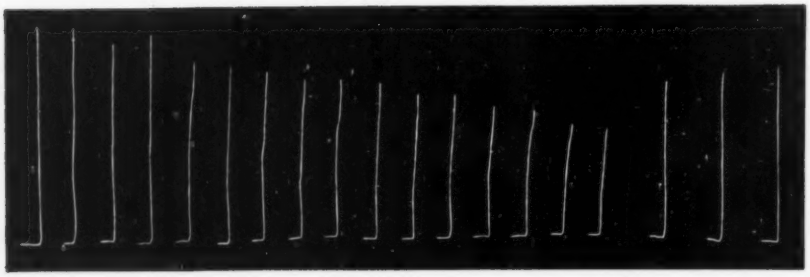

FIG. 3.—Frog rectus abdominis muscle in 10 ml. bath. Effect of repeated doses of 15 μg. of suxamethonium. From the first to the sixteenth dose, a dose cycle of 6 min. (1.5 min. exposure and 4.5 min. recovery) was used. From the sixteenth to the nineteenth dose a 30 min. cycle (1.5 min. exposure and 28.5 min. recovery) was employed.

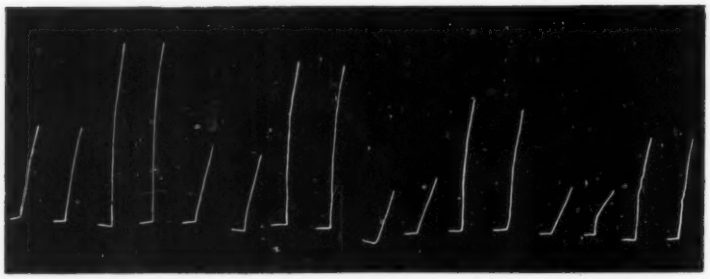

FIG. 4.—Frog rectus abdominis muscle in 10 ml. bath. Effect of repeated alternate pairs of doses of 5.0 μg. and of 7.5 μg. of suxamethonium. A dose cycle of 6 min. (1.5 min. exposure and 4.5 min. recovery) was used throughout.

TABLE I

PROTOCOL OF AN ASSAY OF SUXAMETHONIUM BY MEANS OF THE FROG RECTUS PREPARATION, USING A LATIN SQUARE DESIGN

Values indicate height of response in mm. SL=0.15 ml. and SH=0.20 ml. of 50 μg./ml. solution of standard. TL=0.15 ml. and TH=0.20 ml. of 1 in 2 dilution of test preparation.

	SL	SH	TL	TH	Total
	55	83	49	73	260
	SH	SL	TH	TL	
	78	51	67	39	235
	TL	TH	SH	SL	
	36	60	66	37	199
	TH	TL	SL	SH	
	58	34	38	62	192
Total	227	228	220	211	886

second experiment of the same type yielded similar results. A solution assayed by the rectus method at 29.00 mg./ml. with limits of 27.11 to 31.03 mg./ml. (93.5 to 107.0%) gave a value of 28.25 mg./ml. by the mouse rotating drum method of Collier, Hall and Fieller (1949).

Estimation in Urine and Blood.—Suxamethonium was added to human urine to give a concentration of 10 μg./ml. Assayed against suxamethonium dissolved in Ringer solution, this sample gave a value of 9.10 μg./ml. with limits of 8.21 to 10.01 μg./ml. (90.1 to 110.0%). Suxamethonium was added to eserinized rabbit blood to give a concentration of 10 μg./ml. and assayed against standard suxamethonium in eserinized Ringer solution. This gave a value of 11.14 μg./ml., with limits of 9.81 to 12.64 μg./ml. (88.1 to 113.5%). Both the above assays were performed with 16 dose randomized block designs.

Laudexium

Action of Drug.—Laudexium, like tubocurarine and unlike suxamethonium, failed to stimulate the frog rectus muscle, even when 1 mg. was added to the bath. Laudexium also resembled tubocurarine in antagonizing the stimulant action of acetylcholine and suxamethonium on this preparation. In the experiment illustrated in Fig. 5, 0.5 μg. of laudexium reduced the response to acetylcholine rather more effectively than did an equal dose of tubocurarine. De Jalon (1947) has pointed out that the inhibition by tubocurarine of the response to acetylcholine persists even after washing the tubocurarine out of the bath. A similar effect could be seen after laudexium.

The extent to which a given dose of laudexium reduced the contracture varied with the dose of agonist (acetylcholine or suxamethonium) used. It is evident from Fig. 5 that the lower the dose of agonist, the more sensitive the preparation to laudexium. With too small a dose of agonist, however, repeated responses to a given dose of laudexium proved inconsistent. On the basis of a number of experiments, we decided to use for assay purposes just sub-maximal doses of agonist (1 to 5 μg. of acetylcholine or 3 to 15 μg. of suxamethonium, according to the sensitivity of the preparation).

Arrangement of Doses Within a Cycle.—As already noted, even after laudexium has been washed out of the bath, it depresses the response to agonist. We call this the "hangover" effect. Taking this effect into account, doses within a cycle were arranged in the following sequence: *agonist, wash, laudexium, agonist, wash, agonist,*

TABLE II

EFFECT OF DESIGN ON PRECISION OF ASSAY OF SUXAMETHONIUM BY MEANS OF THE FROG RECTUS PREPARATION

Three designs were used to assay similar solutions.

Design of Assay	Potency (μg./ml.)	95% Fiducial Limits		Slope (b)	Variance (s^2)	Index of Precision (s/b)
		(μg./ml.)	%			
Latin square	92·81	91·23–94·39	98·3–101·7	26·00	1·50	0·047
Randomized block	92·41	87·60–97·40	94·8–105·4	21·38	11·40	0·158
Serially balanced	91·75	86·43–97·44	94·2–106·2	29·83	24·63	0·166

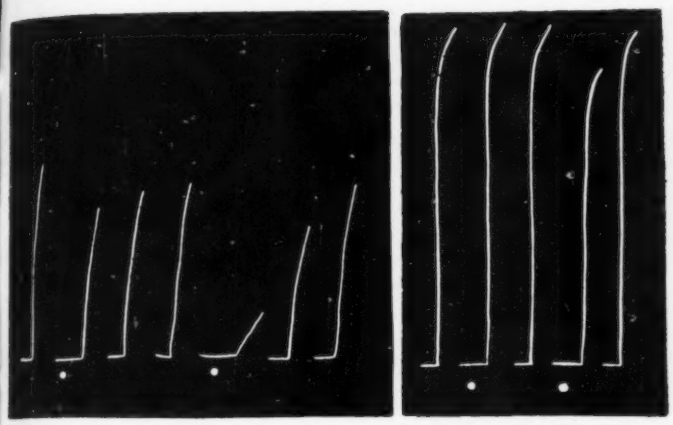

FIG. 5.—Frog rectus abdominis muscle in 10 ml. bath. Successive responses to 2 min. exposure to acetylcholine, **A** to **G**, 1.5 μg. (lever magnification × 15), **H** to **M**, 6 μg. (lever magnification × 5). At white dots at **B** and **J**, 0.5 μg. of tubocurarine and at **E** and **L**, 0.5 μg. of laudexium were added 1.5 min. before acetylcholine.

wash, etc. The sequence *agonist, wash, etc.*, was continued until no further increase in response to agonist could be seen, that is, until the hangover effect was apparently removed. The last response to agonist was termed a stabilized response and became the first dose of the next cycle. If the interpolated doses of agonist were omitted or reduced to one, the response to agonist in the presence of laudexium disappeared after a few doses of laudexium.

Each exposure of the preparation to agonist lasted 1.5 min. Laudexium was added 1.5 or 2 min. before agonist, giving a total exposure to laudexium of 3 or 3.5 min.

Choice of Metameter.—The stabilized response to agonist and the immediately following response to agonist in the presence of laudexium were measured. The % reduction was generally taken as response metameter. We have also used the metameter of response to agonist in the presence of laudexium (or reduced response). The results of using both metameters are compared in Table V and discussed below.

Dose/Response Curve.—When the logarithm of the laudexium dose was plotted against % reduction, we obtained a typical sigmoid curve with a central rectilinear portion, corresponding to a reduction of 20 to 80%. For assay purposes, doses of laudexium were chosen to produce a response within the rectilinear portion.

Using reduced response as alternative metameter, we obtained a sigmoid dose/response curve with a negative slope. The central portion of this curve was also rectilinear within the limits of 80 to 20% response. It was therefore possible to use either metameter in analysing results.

Effect of Fall-off in Response to Agonist.—We have already described the fall-off in response to suxamethonium with repeated doses. Acetylcholine produced the same effect, though to a lesser extent, as can be seen from the totals of columns and rows in Table III. This table also shows that, while the reduced response metameter falls off as the assay proceeds, that of % reduction rises slightly.

Assay Design and Analysis.—Using the above arrangement of doses within a cycle, we adopted a (2+2) pattern of assay with high and low doses of test and standard laudexium. These were given in orders based on a randomized block design (usually 4 × 4) or a 4 × 4 Latin square. The protocol of a typical Latin square assay of laudexium is given in Table III. Table IV contains the analysis of covariance and the results of these analyses are summarized in Table V. Assays usually gave fiducial limits lying within the requirements for tubocurarine of the *British Pharmacopoeia* (1953).

Check on Method.—A solution containing 12 mg./ml. laudexium in Ringer solution assayed at 11.65 mg./ml., with fiducial limits of 11.28 to 12.03 mg./ml. (96.8 to 103.3%). Other experiments

yielded similar results. The solution assaying at 11.65 mg./ml. by the frog rectus gave a value of 11.9 mg./ml. by the mouse rotating drum method.

Estimation in Urine.—We investigated whether the method of Mahfouz (1949) could be applied to laudexium. We added laudexium to human urine to give a concentration of 10 μg./ml., and in one experiment, based on an 8 dose randomized block design, we found 10.86 μg./ml. with limits of 8.80 to 13.85 μg./ml. (78.3 to 127.6%). Two further experiments gave similar results.

TABLE III

PROTOCOL OF AN ASSAY OF LAUDEXIUM BY MEANS OF THE FROG RECTUS PREPARATION USING A LATIN SQUARE DESIGN

ACh = response to 2 μg. of acetylcholine in mm. ACh + La = response to acetylcholine in presence of laudexium in mm. SL = 0.1 ml. and SH = 0.2 ml. of 10 μg./ml. solution of standard preparation. TL = 0.1 ml. and TH = 0.2 ml. of 1 in 2,000 dilution of test preparation. % Redn. = % reduction of response.

	SH	TH	TL	SL	Total
ACh	40	42	39	41	162
ACh + La	17	13	35	30	95
% Redn.	57·5	69·0	10·3	26·8	163·6
	TL	SL	SH	TH	
ACh	38	39	40	36	153
ACh + La	36	32	10	11	89
% Redn.	5·3	17·9	75·0	69·4	167·6
	TH	SH	SL	TL	
ACh	36	35	36	33	140
ACh + La	10	10	31	25	76
% Redn.	72·2	71·4	13·9	24·2	181·7
	SL	TL	TH	SH	
ACh	38	35	34	36	143
ACh + La	32	31	11	10	84
% Redn.	15·8	11·4	67·6	72·2	167·0
Total ACh	152	151	149	146	598
,, ACh + La	95	86	87	76	344
,, % Redn.	150·8	169·7	166·8	192·6	679·9

TABLE V

EFFECT OF CHOICE OF RESPONSE METAMETER AND METHOD OF ANALYSIS ON RESULTS OF TWO ASSAYS OF LAUDEXIUM, USING A LATIN SQUARE DESIGN

Assay No.	Response Meta-meter	Method of Analysis	Potency Ratio	95% Fiducial Limits (Ratio)	95% Fiducial Limits (%)
1	% Reduction	Variance	1·408	1·191–1·664	84·6–118·2
		Covariance	1·443	1·231–1·690	85·3–117·1
	Reduced response	Variance	1·557	1·267–1·914	81·4–122·9
		Covariance	1·329	1·116–1·584	84·0–119·2
2	% Reduction	Variance	0·967	0·879–1·063	90·9–110·0
		Covariance	1·015	0·923–1·117	90·9–110·0
	Reduced response	Variance	1·000	0·917–1·091	91·7–109·1
		Covariance	0·969	0·882–1·067	90·9–110·1

DISCUSSION

Contaminants.—Since choline and succinylmonocholine are possible contaminants of suxamethonium, their very low potency on the frog rectus is important. For example, if as much as 10% succinylmonocholine were present in a specimen of suxamethonium, the sample would assay at 90.1%, as against 90% if the contaminant were inert. With choline, the bias would be even less, and we therefore consider that contamination with these two substances is unlikely to bias estimates of potency.

Response Fall-off and Assay Design.—An assay method is required which allows a sufficiently large number of doses to be fitted into a working day, at the same time compensating for any fall-off by a suitable method of design and analysis.

Fig. 2 shows that fall-off is reduced by extending the recovery period (see, too, Norton and de Beer, 1954). We consider it more efficient, however, to give 16 doses in a 6 min. cycle and

TABLE IV

ANALYSIS OF COVARIANCE OF THE LAUDEXIUM ASSAY PRESENTED IN TABLE III

M.S.′ and *F*′ are mean square and variance ratios of the responses adjusted for covariance. N.S. = Not significant.

Source	d.f.	S.S. (x^2)	xy	S.S. (y^2)	M.S.	F	M.S.′	F'
Preparations	1	9·00	15·8250	27·82563				
Regression	1	0·00	0·0000	11,486·48602				
Parallelism	1	2·25	9·4875	40·00563	40·00563	1·10668 (N.S.)	5·30271	< 1·0 (N.S.)
Doses	3	11·25	25·3125	11,554·31188				
Rows	3	75·25	−45·0125	48·15187	16·05062	0·44401 (N.S.)		
Columns	3	5·25	−31·4875	222·43188	74·14396	2·05106 (N.S.)		
Error	6	12·00	30·5250	216·89375	36·14895		27·84915	
Total	15	103·75	−20·6625	12,041·78938				

Source	Ex^2	Exy	Ey^2	d.f.	Ey'^2	M.S.′
Parallelism	2·25	9·4875	40·00563	1	5·30271	5·30271
Error	12·00	30·5250	216·89375	5	139·24579	27·84915
Parallelism + error	14·25	40·0125	256·89938	6	144·54850	

compensate for fall-off. This becomes particularly important in the laudexium assay when several cycles of *agonist, wash,* may be required to be interpolated between doses of laudexium.

Analysis of the 25-dose serially balanced design of Finney and Outhwaite (1956) failed to reveal a residual effect from a previous dose other than the fall-off described. This is in confirmation of our observations on the assays involving repeated alternate pairs of high and low doses of suxamethonium.

For these reasons we believe that the 4×4 Latin square design is basically the most efficient for the suxamethonium assay, although a somewhat more refined analytical technique than the classical methods for dealing with parallel line assays may be required.

In the laudexium assay we preferred acetylcholine as agonist, because the fall-off was less than with suxamethonium. The response to acetylcholine in the presence of laudexium fell off less than that to acetylcholine alone, both trends being approximately linear over the assay period. This is in further confirmation of the experiment involving repeated alternate pairs of doses of suxamethonium where a similar effect was observed (see Fig. 4). This leads to the slight rise in the percentage reduction metameter already mentioned.

In this assay, as the change in response with time is only relatively slight we have found it advantageous to use a 4×4 randomized block design. In some earlier assays, we obtained rather wide fiducial limits and for a time changed over to Latin square design obtaining better limits. We now feel that this improvement was coincidental and have adopted randomized blocks as a routine.

In a few laudexium assays we attempted to reduce further the error variance of the Latin square by covariance analysis, using the stabilized response to acetylcholine as concomitant variate. We found no consistent gain in precision (Table V) and, in view of the computational labour involved, do not use this method as a routine.

The alternative metameter of reduced response, previously defined for this assay, showed considerable fall-off with time. Using this metameter and applying covariance analysis with the same concomitant variate as above we obtained in some assays a slight gain in precision and rather wider limits in others (Table V).

For the above reasons we have adopted the percentage reduction in response as metameter and are now using randomized block design. As in the suxamethonium assay it may be advantageous to use slightly more rigorous methods of analysis than the classical methods for parallel line assays. These will be discussed in a later publication.

Hangover in the Laudexium Assay.—As we have described, the hangover effect can be largely overcome by interpolating doses of agonist after laudexium has been washed from the bath. Since recovery from hangover operates in the opposite direction to fall-off, the choice of a stabilized response to agonist depends to some extent on the operator. We therefore sought an alternative method of compensating for hangover. One such method would be to omit interpolated doses of agonist and to apply the serially balanced design of Finney and Outhwaite (1956). As we have already described, omission of interpolated doses of agonist leads to disappearance of the response in the presence of laudexium and therefore this method could not be used.

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INCREASE OF 5-HYDROXYTRYPTAMINE IN THE RAT BRAIN BY RAUNESCINE

BY

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The Rauwolfia alkaloid raunescine (5 mg./kg., intraperitoneally) increased the concentration of 5-hydroxytryptamine in the brains of rats after iproniazid pre-treatment. This was evident 3 to 4 hr. after raunescine administration. There was no general increase in the noradrenaline content of the brains. In the intestine, raunescine depleted the 5-hydroxytryptamine content by about 50% within 3 to 4 hr. if the animals had been pre-treated with iproniazid. Iproniazid did not increase the content of noradrenaline in the intestine.

When raunescine or reserpine are administered to rats the noradrenaline content of brain and intestine is decreased more rapidly and probably more completely than the 5-hydroxytryptamine content (Paasonen and Dews, 1958; Paasonen and Kärki, 1958; Kärki and Paasonen, 1959). This difference is particularly clear in the intestine. To find out whether the greater depletion of noradrenaline can be explained by destruction of the liberated amine by monoamine oxidase, we treated the rats with iproniazid (Zeller, Barsky, Fouts, Kirchheimer and van Orden, 1952), before administering raunescine. Iproniazid increases the content of 5-hydroxytryptamine and noradrenaline of the brain in various species (Udenfriend, Weissbach and Bogdanski, 1957; Pletscher, 1956a and b, 1957; Paasonen and Giarman, 1958) and also reduces the depletion due to reserpine of these amines in the brain, heart and adrenals (Carlsson, Rosengren, Bertler, and Nilsson, 1957; Pletscher, 1957; Brodie and Shore, 1957).

METHODS

White female rats weighing 120 to 160 g. were used. The animals were not fasted. The experiments were carried out at room temperature (20 to 23°). The whole brain (excluding cerebellum) and about 1 g. of intestine (duodenum and upper jejunum) were taken from each rat. The tissues from two animals were always combined and one half was used for noradrenaline and the other half for 5-hydroxytryptamine determination. The dissection and extraction procedure for noradrenaline was the same as that used in previous work (Kärki and Paasonen, 1959).

For the extraction of 5-hydroxytryptamine the acetone-heptane method (Kärki and Paasonen, 1959) was used. This is essentially similar to the method suggested by Shore and Olin (1958) for catechol amine extraction, with the difference that acetone was used instead of butanol. The tissues were first ground with two volumes 0.01 N-HCl and sand, then extracted with 90% (v/v, final concentration) acetone, containing 4 g. NaCl. After shaking for 1 hr. the aqueous phase was separated by the addition of heptane. This phase still contained some acetone which was evaporated *in vacuo* at 35°. With this method, the recoveries of added 5-hydroxytryptamine have been 80% or more. No correction was made for the loss of 5-hydroxytryptamine due to the extraction method.

The blood was collected after decapitation in tubes containing heparin and extracted with 95% acetone (Amin, Crawford, and Gaddum, 1954).

The amines were assayed biologically by using the cat blood pressure method for noradrenaline (Kärki, 1956) and the rat stomach method of Vane (1957) for 5-hydroxytryptamine.

Iproniazid phosphate (Hoffmann-La Roche) was injected subcutaneously in a dose of 100 mg./kg. of the base. In each experiment, all the animals were injected with iproniazid at the same time. Raunescine was administered from 14 to 17 hr. after iproniazid and the animals were then kept for various periods—up to 6 hr.—before they were killed. Control animals treated with iproniazid alone were kept alive on the average for the same period of time. The survival times after iproniazid of the control and experimental animals differed by not more than 2 hr. Since the brain 5-hydroxytryptamine and noradrenaline remain elevated after iproniazid for at least 48 hr. (Spector, Prockop, Shore, and Brodie, 1957; Paasonen and Giarman, 1958) it seemed unlikely that differences of 2 hr. in survival time after iproniazid would have been significant.

The raunescine (S. B. Penick & Co., N.Y.) was dissolved in glacial acetic acid and the solution diluted so that the final concentration of acetic acid

TABLE I

EFFECTS OF IPRONIAZID AND RAUNESCINE ON 5-HYDROXYTRYPTAMINE AND NORADRENALINE CONTENT OF THE BRAIN AND SMALL INTESTINE OF THE RAT

All values are ng./g. wet weight. Each column contains results obtained on the same day. Iproniazid (100 mg./kg. subcutaneously) was injected about 18 hr. before killing the animals. The time interval is that between the injection of raunescine (5 mg./kg. intraperitoneally) and the removal of the tissues.

Drug	Time (hr.)	5-Hydroxytryptamine								Mean	Noradrenaline								Mean
Brain																			
None		248	279	213	420	340	457	332	316	326	140	140	230	340	169	326	323	200	234
Iproniazid		526	564	315	1,320	1,050	1,130	1,060	790 588	816	274	273	543	615	315	410	540	475	443
„ +raunescine	1				857		840	1,040	550 670	791				385		245	600	436	417
„ „	2				1,340				790 740	957				355					355
„ „	3				2,250		1,300	1,200	980 870	1,103				490		208	370	405	344
„ „	4	778	770	795		980					258	263	303	495					
„ „	6						990	569	842	800						181	625	342	383
Intestine																			
None		2,130	2,250	5,000	3,200	4,530		6,200	4,320	3,950	243	250	180	210	255		410	270	260
Iproniazid		4,350	6,600	4,500	4,620	7,060		7,330	6,110	5,800	460	280	155	235	161		217	195	232
„ +raunescine	1				4,940			5,520	4,740	5,070				270			113	80	154
„ „	2				2,680					2,680				128					128
„ „	3				2,810			5,000	3,260	2,970				93			72	82	62
„ „	4	2,270	1,880	1,050		4,500					85	48	39		41				
„ „	6							7,250	4,630	5,940							57	37	47

injected was not in excess of 2%. The dose of raunescine was 5 mg./kg. and it was given intraperitoneally in a solution containing 2.5 mg./ml. 5-Hydroxytryptamine was used as creatinine sulphate (Abbott Lab., Chicago, Ill.) and noradrenaline as bitartrate (Bayer Products, London). In each case the dose refers to the base.

RESULTS

The detailed results on brain and intestine are presented in Table I. For unknown reasons the scatter in the control groups was rather great, but values obtained at the same time and under strictly identical conditions were comparable. In three pairs of control rats, taken at the same time, the highest value was in no case more than 10% higher than the lowest. Consequently results obtained on the same day are shown in the same columns. The mean % changes are shown in Figs. 1 and 2 and their statistical significance is indicated in the text.

Brain

5-Hydroxytryptamine. — Iproniazid treatment increased the 5-hydroxytryptamine content in brain ($P<0.001$), but there was some indication that raunescine, when allowed to act for 1 hr., lowered the 5-hydroxytryptamine content below that of the iproniazid controls. After 3 to 4 hr., however, there is a mean increase of 44%, which was statistically significant as compared with the control ($P<0.05$). The values obtained 6 hr. after injection of raunescine indicate that the content of 5-hydroxytryptamine had fallen to the pre-injection level or below (Fig. 1).

These findings are in marked contrast to those obtained with raunescine without iproniazid pre-treatment when a dose of 5 mg./kg. of raunescine lowered the 5-hydroxytryptamine content by some 40% within 4 hr. (Kärki and Paasonen, 1959).

Noradrenaline.—Iproniazid increased the amount of noradrenaline in brain ($P<0.01$). After raunescine there was a decrease of about 20% of the amine as compared with controls treated with iproniazid alone, but the difference was not statistically significant.

Intestine

5-Hydroxytryptamine.—In the intestine the % increase of the 5-hydroxytryptamine content by iproniazid was not as great as that in brain, but it was statistically significant ($P<0.05$). After raunescine, the 5-hydroxytryptamine content was decreased compared with controls treated with iproniazid alone. The reduction was statistically significant ($P<0.01$) 3 to 4 hr. after the administration of raunescine.

Noradrenaline.—Iproniazid produced no significant change in the noradrenaline content of the intestine, but raunescine given after iproniazid produced a considerable depletion of noradrenaline ($P<0.01$).

Blood

5-Hydroxytryptamine.—In three experiments the blood from normal rats, rats treated with iproniazid and those treated with iproniazid plus

FIG. 1.—Mean % changes (derived from Table I) in 5-hydroxytryptamine (5-HT), ●——●, and noradrenaline, O——O, content of brain caused by raunescine in rats pre-treated with iproniazid.

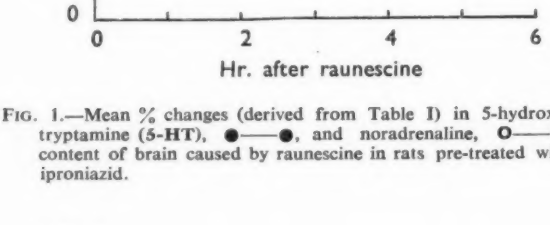

FIG. 2.—Mean % changes in 5-hydroxytryptamine (5-HT), ●——●, and noradrenaline, O——O, content of intestine caused by raunescine in rats pre-treated with iproniazid.

raunescine (1, 3 and 6 hr.) was analysed. The following is a typical example. Content of 5-hydroxytryptamine (ng./ml.) in untreated control, 210; after iproniazid, 504; after iproniazid plus raunescine, at 1 hr. 240, at 3 hr. 130, and at 6 hr. 93.

Symptoms Observed

Iproniazid alone increased motor activity in rats. This was accompanied by slight piloerection, exophthalmos and mydriasis. Raunescine increased all these symptoms. The effect was clear within 5 to 10 min. and lasted for the whole observation time. However, there was usually some indication that the excitement and other symptoms were decreased 5 to 6 hr. after the injection of raunescine.

DISCUSSION

These experiments show that 3 to 4 hr. after administration of raunescine in iproniazid pre-treated rats there was a significant increase in brain 5-hydroxytryptamine. A possible explanation of this increase is that 5-hydroxytryptamine, which is located in intracellular granules (Blaschko, 1957) is normally destroyed by monoamine oxidase when the former is released from its binding sites. Iproniazid prevented this destruction and we may assume that the cells were trying to replace the "vacant" space in the granules by synthesizing more 5-hydroxytryptamine, thus increasing the total content of this amine.

Another possible explanation of the increase in 5-hydroxytryptamine is that the brain may be able to pick up some of the 5-hydroxytryptamine liberated from other tissues. The blood findings, however, showing a consistent fall in iproniazid-raunescine animals, did not support this explanation. On the other hand, the platelets become unable to bind 5-hydroxytryptamine (Shore, Pletscher, Tomich, Kunzman, and Brodie, 1956), which may be of significance. We are establishing whether the 5-hydroxytryptamine injected into rats will penetrate the brain tissue more easily after raunescine.

In the intestine, there was no evidence of an increase of 5-hydroxytryptamine after raunescine in the iproniazid-treated animals. It is interesting and relevant to note that raunescine was able to release about half the intestinal 5-hydroxytryptamine after iproniazid, although it is almost inactive in this respect in animals which have not been pre-treated with iproniazid (Kärki and Paasonen, 1959).

Apart from a few estimations, there was no indication of elevated levels of noradrenaline after raunescine in iproniazid-treated rats. This agrees with the findings of Pletscher (1957) that iproniazid increases the content of 5-hydroxytryptamine in rat brain more than the content of noradrenaline and suggests that the rôle of amine oxidase may be more significant in the katabolism of 5-hydroxytryptamine than it is in that of noradrenaline (see Corne and Graham, 1957).

We may assume that the mechanism responsible for the elevation of brain 5-hydroxytryptamine after raunescine in rats pre-treated with iproniazid also works in animals not treated with iproniazid. The increase of 5-hydroxytryptamine may be masked by the simultaneous depletion of the amine due to the rauwolfia alkaloid. It has previously been shown that noradrenaline is

released more completely than 5-hydroxytryptamine from the brains of rats after reserpine and raunescine (Paasonen and Dews, 1958; Kärki and Paasonen, 1959). The brain is thus differentially depleted of the two amines in both normal and iproniazid-treated rats.

When raunescine is administered alone, it produces sedation and lowers the content of noradrenaline in the brain even when there is no demonstrable change in 5-hydroxytryptamine in this organ (Kärki and Paasonen, 1959). On the other hand, when raunescine is administered in the presence of iproniazid it stimulates, as shown both in the present experiments and in those of Brodie and Shore (1957) on rabbits, and increases 5-hydroxytryptamine content. We do not know whether this increase represents the "free" or "bound" amine as defined by Brodie (1958). It is conceivable that in each case the pharmacological effects are produced by free 5-hydroxytryptamine (that which has been released from its binding sites into the cytoplasm). It would then follow that low concentrations of free 5-hydroxytryptamine are depressant while high concentrations are stimulant as suggested by Brodie (1958). There is, at present, no direct evidence for this theory.

This study leaves unanswered the question whether or not noradrenaline and/or 5-hydroxytryptamine is the most important mediator in the action of the rauwolfia alkaloids.

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THE COMPARATIVE TOXICITY OF ANTIBIOTICS TO SKIN

BY

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The toxicity of sixteen antibiotics to skin *in vitro* was investigated by a microrespirometer technique and by the histological appearance of 3-day cultures. There was close agreement between the results obtained by the two methods and between tests with human and with guinea-pig skin. Antibiotic concentrations which caused more than 30% respiratory damage after one day invariably produced abnormal cultures, and more than 60% respiratory damage often resulted in necrotic cultures. Dose/response curves were obtained for the effect of antibiotics upon skin respiration. Mathematical evaluation had little advantage over visually fitted dose/response curves. The ED50 provided a reproducible measure of the relative toxicity of different antibiotics to skin, but the ED30 probably provided a better comparison because this was the highest concentration of antibiotic which permitted migration of epithelium. Many antibiotics at therapeutic concentrations show little toxic effect upon skin *in vitro*. It is suggested that the healing of surface lesions might well be retarded if the antibiotic concentration found toxic *in vitro* were maintained at a similar concentration *in vivo*.

As high concentrations of antibiotics are frequently applied on surface wounds such as burns, it is desirable to know the local toxicity of the antibiotics, since impairment of wound healing could possibly offset advantages gained by elimination of infecting micro-organisms.

Tissue culture offers a convenient approach to this problem and was used by Cruickshank and Lowbury (1952) to assess the effect of some antibiotics on the migration of epithelium around small explants of human skin. Hu, Livingood, and Hildebrand (1956) used a roller tube technique to study the effect of some antibiotics upon human skin cells. Other workers have used other culture materials; Lépine, Barski, and Maurin (1950) used chick-embryo cultures for tests on the toxicity of chlortetracycline and chloramphenicol, and Schrek (1953) tested oxytetracycline on rat thymus cells.

Though clinical conditions are doubtless different from those of *in vitro* experiments, cultures of adult mammalian skin are obviously more appropriate to this problem than chick-embryo cultures and the technique of skin culture used by Cruickshank and Lowbury (1952) is somewhat analogous to conditions found in a healing surface wound. However, the use of histological criteria in this method has the disadvantage that many tests are necessary in order to determine the maximum tolerable antibiotic concentration. In the present investigation a microrespirometer technique (Cruickshank, 1954) has been applied to this problem and the results have been compared with those obtained by the histological method (Cruickshank and Lowbury, 1952). This gives a rapid and reproducible method for determining the immediate toxicity of antibiotics to skin.

METHODS

Guinea-pig and human skin slices were used in this investigation; guinea-pig skin was cut free-hand from the dorsum of the ear (Cruickshank, 1954), human skin was that left over after the grafting of burns. Since human skin obtained in this manner has been previously found to be less reliable for metabolic studies than guinea-pig skin owing to variations in thickness and the amount of keratin present (Lawrence and Ricketts, 1957), preliminary experiments with guinea-pig skin were followed by experiments with human skin.

The following standard skin culture medium was used for all the experiments: homologous serum 5 parts; Krebs-Ringer phosphate 3 parts; 2% glucose 1 part; dihydrostreptomycin sulphate 500 μg./ml. 1 part (Cruickshank and Lowbury, 1952).

For toxicity tests antibiotics were dissolved in physiological saline at 10 times the required strength. Where necessary, the *p*H was adjusted to 7.0. These solutions were incorporated into tissue cultures by making 1:10 dilutions with skin culture medium. Sparingly soluble materials were employed as

suspensions; these were agitated violently immediately before use so that the suspension was as uniform as possible.

The differential microrespirometer described by Cruickshank (1954) was used for skin respiration studies. With this device aerobic respiration of small skin slices could be measured accurately over long periods of time. Test solutions were added after respiration measurements had been made for 2 hr. Readings were then taken for the next 4 to 5 hr., discontinued at night (about 15½ hr.), recommenced the following day and continued until 24 hr. after addition of the test solution. The mean respiratory rate of the 22 to 24 hr. period was then expressed as % of the 2 hr. control period before addition of the test material. It was found convenient to terminate experiments at 24 hr., because control cultures respired steadily over this period; some decrease in respiration occurred over longer time periods. Respiratory damage caused by toxic agents after 48 hr. was proportional to that occurring after 24 hr.

A preliminary experiment on each antibiotic discovered the approximate concentrations producing slight and severe inhibition of respiration after 24 hr. Having established this range, a series of intermediate concentrations were prepared and tested. A control culture in which saline was added to the respirometer was always included with every experiment.

Three-day cultures of skin were prepared in the manner described by Cruickshank (1951). Antibiotic concentrations causing about 5%, 25%, 50% and 95% respiratory damage were chosen. Histological appearance of these cultures was classified into the four groups defined by Cruickshank and Lowbury (1952): (*a*) Normal cultures: epithelium migrates over the cut surface of the dermis so that the explant resembles a "cyst" of dermis within epidermis. (*b*) Minor inhibition: these stain normally but epithelial migration is less than in normal cultures, usually only around the "shoulders" of the explant. (*c*) Severe inhibition: these cultures also stain normally but no migration of epithelium occurs. (*d*) Necrotic cultures: these stain normally and, in some instances, the explant is destroyed.

RESULTS

Respiration Studies.—Sixteen antibiotics were tested by the same experimental technique. To illustrate the procedure, results obtained with vancomycin (McCormick, Stark, Pittenger, Pittenger, and McGuire, 1955–56) will be quoted in full.

Fig. 1 shows the respiratory changes of guinea-pig skin caused by various concentrations of vancomycin. Table I shows the corresponding % respiratory damage of guinea-pig and human skin. No change in respiration could be detected until the second day. This "delayed" toxic action was common to all antibiotics tested except novobiocin; toxic concentrations of this substance caused an appreciable decrease in respiration 2 hr. after addition. With the other antibiotics some toxic effects could be discerned 8 to 10 hr. after addition and by 16 hr. toxic

TABLE I

THE RESPIRATORY DAMAGE TO SKIN CAUSED BY VANCOMYCIN

Drug Conc. (mg./ml.)	Guinea-pig				Human		
	O_2 Uptake (µl./mg./hr.)		% Respiratory Damage	Curves in Fig. 1	O_2 Uptake (µl./mg./hr.)		% Respiratory Damage
	0 to 2 hr. Before	22 to 24 hr. After			0 to 2 hr. Before	22 to 24 hr. After	
0	0·96	0·97	0	Not shown	0·36	0·38	0
1·0	1·00	0·95	5	A	0·42	0·39	7
2·5	0·99	0·89	10	B	0·40	0·34	15
5·0	0·89	0·74	17	C	0·32	0·25	22
7·5	1·01	0·77	24	D	0·35	0·24	31
10·0	1·00	0·47	53	E	0·44	0·18	59
20·0	1·05	0·37	65	F	0·41	0·11	75

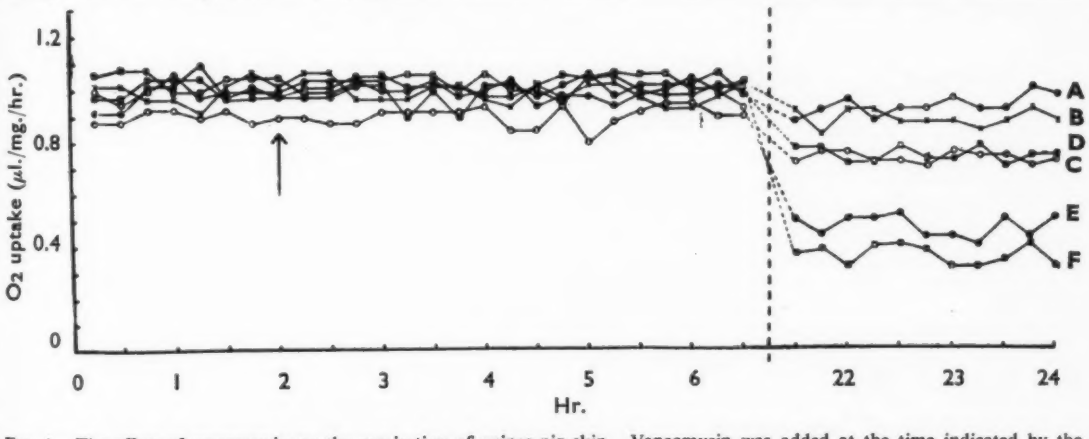

FIG. 1.—The effect of vancomycin on the respiration of guinea-pig skin. Vancomycin was added at the time indicated by the arrow in the following concentrations: A, 1.0 mg./ml.; B, 2.5 mg./ml.; C, 5.0 mg./ml.; D, 7.5 mg./ml.; E, 10.0 mg./ml.; F, 20.0 mg./ml. The broken vertical line denotes the overnight period during which observations were discontinued.

effects were obvious. These respiratory changes reached a maximum 18 to 20 hr. after the antibiotic had been added to the culture. None of the antibiotics caused any initial stimulation of respiration, in contrast to observations made with certain other bacterial products (Lawrence, 1958, 1959).

Log drug concentration plotted against % respiratory damage gave a sigmoid curve and a linear dose/response curve was obtained by transferring the observed % to a probability scale. Fig. 2 shows the dose/response curve for vancomycin.

The relative toxicities of antibiotics to skin were assessed by comparing the median effective concentration (ED50) of each antibiotic with the ED50 of penicillin. Penicillin was chosen as the standard with a toxicity of 1.0 because it is one of the least toxic substances to skin so far discovered. Table II shows the relative toxicity indices of the antibiotics computed on weight/unit volume and molar bases. It is important to note that these indices are valid only for the 50% effect; it is only when the slopes of the dose/response curves are the same that the relative toxicity index is constant for all percentage effects. The slope values of these curves were calculated from the expression (Gaddum, 1948), slope $\simeq 1/\log.ED69 - \log.ED31$, and have been included in Table II for comparison since it is generally accepted that substances producing dose/response curves with similar slopes probably have comparable modes of action (Finney, 1947; Gaddum, 1948).

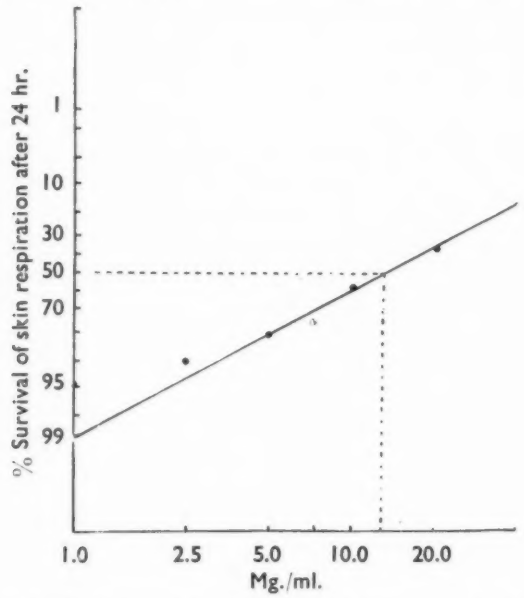

FIG. 2.—Dose/response curve for the effect of vancomycin on skin respiration. M.W. of vancomycin is 3,350 (approx.). ED50 13 mg./ml. (38.8×10^{-5} M). Ordinate, probability scale.

TABLE II

CONCENTRATIONS OF ANTIBIOTICS REQUIRED TO REDUCE THE RESPIRATION OF SKIN BY 50% AFTER 24 HR.

The dose/response curves of antibiotics marked with an asterisk were extrapolated because of their low solubility.

Antibiotic	M.W.	ED50 (mg./ml.)	Toxicity Relative to Penicillin	ED50 (M × 10^{-5})	Molar Toxicity Relative to Penicillin	Slope of Dose/Response Curve
Penicillin	356	7·4	1·0	208	1·0	2·78
Cephalosporin N	359	23·4	0·32	652	0·32	0·75
Vancomycin	3,350	13	0·6	38·8	5·4	2·63
Oleandomycin*	828	36·3	0·16	438	0·47	2·08
Erythromycin*	725	4·6	1·6	63·5	3·3	2·33
Carbomycin	953	0·26	28·5	27	77	5·88
Streptomycin	964	7·8	0·95	80·9	2·6	1·54
Neomycin	?1,000	4·4	1·7	4·4	4·7	1·23
Chloramphenicol	323	8·7	0·85	269	0·77	0·69
Chlortetracycline	514	0·25	29·6	4·9	42·4	2·63
Oxytetracycline	481	0·3	24·7	6·2	33·6	2·70
Tetracycline	465	0·26	28·5	6·0	34·7	2·58
Polymyxin B	1,280±70	0·4	18·5	2·9	71·7	1·49
Bacitracin	>1,500	10 mg./ml. non-toxic				
Neopumilin	>2,000	Saturated solution non-toxic				
Novobiocin	612	0·3	24·7	4·9	42·4	2·94

Dose/response relations for the tetracycline antibiotics were also evaluated by the mathematical method of Finney (1947) and compared with the results obtained by the method of Gaddum (1948). This comparison is shown in Table III.

TABLE III

COMPARISON OF RELATIONSHIPS BETWEEN DOSE AND RESPONSE FOR THE TETRACYCLINE ANTIBIOTICS OBTAINED BY DIFFERENT METHODS OF CALCULATION

F: method of Finney (1947). G: method of Gaddum (1948).

Antibiotic	F ED50 (mg./ml.)	Slope	G ED50 (mg./ml.)	Slope
Chlortetracycline ..	0·22±0·07	2·3	0·25	2·6
Oxytetracycline ..	0·32±0·08	2·4	0·30	2·7
Tetracycline	0·25±0·12	2·4	0·26	2·6

Both methods gave similar values for the ED50 and slope. Since the two methods were in such good agreement, visually fitted lines were used in this investigation.

Three-day Cultures.—The histological appearance of human and guinea-pig skin explants cultured in the presence of various antibiotics for 3 days is recorded in Table IV. The toxicity to skin of both species of all the antibiotics tested was found to be similar; these results have

TABLE IV

THE EFFECT OF ANTIBIOTICS ON 3-DAY SKIN CULTURES
Results marked with an asterisk should be read in conjunction with Table V.

Antibiotic	Conc. (mg./ml.)	Histological Appearance						% Resp. Damage after 24 hr.
		No. of Expts.	No. of Explants	Cultural Appearance				
				Normal	Minor Inhibition	Severe Inhibition	Necrosis	
Controls	—	27	54	49	5	0	0	0
Penicillin	1·0	2	4	4	0	0	0	2
	2·5	2	4	2	2	0	0	10
	5·0	3	6	1	2	2	1	32
	10·0	2	4	0	0	0	4	65
Vancomycin	1·0	2	4	4	0	0	0	5
	5·0	2	4	3	1	0	0	15
	10·0	2	4	0	2	2	0	38
	25·0	2	4	0	0	2	2	70
Oleandomycin	10·0	2	4	3	1	0	0	14
	20·0	4	8	5	2	1	0	30
Erythromycin	1·0 (sat. soln.)	4	8	6	2	0	0	10
Streptomycin	1·0	2	4	4	0	0	0	9
	2·5	2	4	2	2	0	0	23
	10·0	2	4	0	0	1	3	64
Neomycin*	2·5	2	4	0	3	1	0	38
Tetracycline	0·1	2	4	2	1	1	0	18
	0·25	2	4	1	1	2	0	48
	0·5	2	4	0	0	2	2	74
	1·0	2	4	0	0	1	3	90
Oxytetracycline*	0·3	2	4	0	0	3	1	50
Polymyxin B*	0·5	2	4	0	1	2	1	56
Bacitracin	10·0	5	10	9	1	0	0	0
Neopumilin	0·01 (sat. soln.)	4	8	6	2	0	0	0
Novobiocin	0·1	2	4	3	1	0	0	10
	0·2	2	4	1	3	0	0	30
	0·5	2	4	0	1	2	1	74
	1·0	2	4	0	0	0	4	93

therefore been grouped together. The % respiratory damage of skin cultured for 1 day in these antibiotic concentrations has also been included in Table IV for comparison.

Neopumilin, oleandomycin, and erythromycin were tested as saturated solutions because of their low solubility. It was not possible to investigate the effect of cephalosporin N or carbomycin upon 3-day cultures of skin.

DISCUSSION

The histological appearance of 3-day skin cultures made in the presence of antibiotics bore a close relationship to the amount of respiratory damage to skin after 1 day (Table IV). Concentrations of antibiotics causing more than 30% respiratory damage invariably produced abnormal 3-day cultures and 60% (or more) respiratory damage was usually associated with necrotic cultures. Antibiotic concentrations causing less than 30% respiratory damage did not generally interfere with the migration of epithelium around skin explants cultured for 3 days. Similarly, the earlier histological results of Cruickshank and Lowbury (1952) can be compared with the respiration results reported in this study (Table V).

TABLE V

THE EFFECT OF ANTIBIOTICS UPON THE CULTURAL APPEARANCE OF SKIN; THE CULTURAL APPEARANCE REPORTED BY CRUICKSHANK AND LOWBURY (1952) COMPARED WITH % RESPIRATORY DAMAGE

Antibiotic	Conc. (mg./ml.)	Histological Results						% Resp. Damage after 24 hr.
		No. of Expts.	No. of Explants	Cultural Appearance				
				Normal	Minor Inhibition	Severe Inhibition	Necrosis	
Neomycin	0·2	1	3	3	0	0	0	5
	1·0	1	3	3	0	0	0	20
	10·0	1	3	0	0	0	3	66
Chloramphenicol	0·1	2	5	3	1	1	0	13
	0·2	1	3	1	1	1	0	18
	1·0	2	6	1	3	1	1	30
	2·0	3	10	5	2	2	1	36
Chlortetracycline	0·2	1	3	3	0	0	0	40
	1·0	1	3	1	2	0	0	90
	2·0	2	6	0	3	3	0	99
Oxytetracycline	0·02	1	3	3	0	0	0	<1
	0·2	1	3	0	3	0	0	30
	1·0	1	3	0	0	0	3	90
Polymyxin B	0·2	2	6	6	0	0	0	33
	1·0	1	3	0	1	0	2	73
	2·0	3	9	0	1	5	3	85

Penicillin and chloramphenicol were exceptional in that concentrations which caused about 40% respiratory damage frequently resulted in severely inhibited or necrotic cultures. The reason for this anomaly is not yet clear; possibly some delayed secondary effect occurs. In contrast Cruickshank and Lowbury (1952) found that high concentrations of chlortetracycline led to inhibition but not necrosis of cultures; the present study showed that similar concentrations of this antibiotic caused extreme respiratory damage (>90%). This may have been due to the fact that chlortetracycline rapidly decomposes at 37°, or the antibiotic may perhaps act as a fixative, like formaldehyde.

These observations suggest that the antibiotic concentration which skin can tolerate without undue interference in the normal metabolic processes is the concentration which produces 30% respiratory damage after one day. Table VI

TABLE VI

THE TOXICITY OF ANTIBIOTICS TO SKIN. AT THESE CONCENTRATIONS, NAMELY THOSE PRODUCING 30% INHIBITION OF RESPIRATION AFTER 24 HR., THE MIGRATION OF EPITHELIUM IS JUST POSSIBLE

The dose/response curves of the antibiotics marked with an asterisk were extrapolated because of their low solubility.

Antibiotic	ED30 (mg./ml.)	Toxicity Relative to Penicillin	ED30 (Molar $\times 10^{-5}$)	Molar Toxicity Relative to Penicillin
Penicillin	5·0	1·0	140	1·0
Cephalosporin N ..	4·0	1·25	111	1·26
Vancomycin ..	8·1	0·62	24·2	5·8
Oleandomycin* ..	20·0	0·25	242	0·58
Erythromycin* ..	2·5	2·0	34	4·1
Carbomycin ..	0·19	26	2·0	70
Streptomycin ..	3·6	1·4	37	3·8
Neomycin ..	1·7	2·9	17	8·2
Chloramphenicol ..	4·0	1·25	124	1·13
Aureomycin ..	0·16	31	3·1	45
Terramycin ..	0·20	25	4·2	33
Tetracycline ..	0·14	36	3·0	47
Polymyxin B ..	0·17	29	1·3	108
Bacitracin	10 mg./ml. non-toxic			
Neopumilin ..	Saturated solution non-toxic			
Novobiocin ..	0·20	25	3·3	42

shows the relative toxicity of the antibiotics at this level (ED30). A comparison of this table with Table II shows that the relative toxicity of the antibiotics differs with the threshold selected (ED50 or ED30); for instance, cephalosporin N is less toxic than penicillin at the ED50 but more toxic at the ED30.

The dose/response curves of the related antibiotics of the tetracycline group (which share similar chemical structure and antibacterial action) had similar slopes. Erythromycin, oleandomycin, and carbomycin also have similar antibacterial activities (Garrod, 1957). Of these, erythromycin and oleandomycin appeared to have comparable effects on skin cultures (slopes 2.3 and 2.1 respectively); but carbomycin was very different (slope 5.9). On structural grounds cephalosporin N (aminoadipic acid penicillin) might have been expected to resemble penicillin in its action on skin, but the penicillin/dose response curve (slope 2.8) was very different from that of cephalosporin N (0.8). This is consistent with certain differences in the range of sensitivity of bacteria to these antibiotics (Newton and Abraham, 1954). It is also possible that the sample of cephalosporin N employed contained impurities which interfered with the test.

Though a similarity in the skin toxicity dose/response curves of two antibiotics may suggest a close relationship in the antibacterial mechanism or chemical structure, this is not necessarily so. For example, vancomycin has a similar slope to that of the tetracycline group (2.6), but bacterial cross-resistance between vancomycin and the tetracyclines has not been encountered (Ziegler, Wolfe, and McGuire, 1955–56). Similarly, polymyxin B and streptomycin share similar slopes (1.5), but their effect on isolated cells is known to be dissimilar. Streptomycin causes inhibition of oxalacetate-pyruvate condensation (Umbreit and Tonhazy, 1949) while polymyxin B is a surface active agent (Newton, 1953). The difference in chemical structure of these two antibiotics is well known. Confirmatory evidence from other sources would seem advisable before drawing conclusions on similarity of toxic action to skin.

The antibiotics may be classified into groups on the basis of their molar toxicities to skin (Table VI). Erythromycin, oleandomycin, bacitracin, and neopumilin are "safe" antibiotics as far as skin is concerned, saturated solutions having little toxic effect.

One large group, including cephalosporin N, vancomycin, chloramphenicol, streptomycin, and neomycin, has a toxicity index of the same order as penicillin (between 0.5 and 8.0). At therapeutic concentrations these materials have little appreciable toxicity towards skin.

The tetracyclines and novobiocin with relative toxicity indices between 35 and 45 form a third group which is somewhat more toxic than penicillin.

Polymyxin B and carbomycin have high indices, of 108 and 70 respectively. However, even these antibiotics have a low toxicity compared with a substance like mercuric chloride which is 424 times as toxic as penicillin (Lawrence, 1958).

The antibiotic concentrations permitting normal skin cultures (Table VI) are generally comparable with such similar values as are available from other workers. Hu *et al.* (1956) obtained the following minimum inhibitory concentrations for antibiotics on human skin *in vitro*: penicillin 1.8 mg./ml.; neomycin 1.0 mg./ml.; chlortetracycline 0.2 mg./ml.; oxytetracycline 0.1 mg./ml. These values, except that quoted for penicillin, agree well with those in Table VI; the present experiments suggest that skin can tolerate 5 mg./ml. penicillin without undue toxic effects.

Schrek (1953) demonstrated 20% mortality of rat thymus cells *in vitro* after 20 hr. in the presence of 0.125 mg./ml. oxytetracycline; the dose/response curve for oxytetracycline indicated that this concentration would be expected to reduce skin respiration by 15% after 1 day.

Lépine *et al.* (1950) using chick embryo fibroblasts found 0.1 mg./ml. chloramphenicol

"quite toxic" and 0.1 mg./ml. chlortetracycline "very toxic." The current investigation showed that 4.0 mg./ml. chloramphenicol and 0.16 mg./ml. chlortetracycline permitted normal skin cultures. The difference between the observations could be due to the diverse nature of the tissues used in the two tests.

Care is needed in applying these results for clinical purposes. Salle, McOmie, and Schechmeister (1937) consider that some form of therapeutic index is desirable in connexion with the use of antiseptics on surface wounds. Such an index is probably of greatest value when comparing substances lethal to bacteria and tissues at similar ranges of concentration. With antibiotics this method seems less appropriate; the enormous difference in susceptibility of bacteria and tissues to antibiotics leads to indices of quite a different order. A more important objection is based on the variations between strains and especially the existence of resistant bacteria which would render such an index invalid. Moreover, variation in susceptibility of bacterial strains to antibiotics makes the application of a single index for a single bacterial species impracticable. It seems, therefore, more useful to have a measure of the tissue toxicity of the antibiotics which can be used together with information on the sensitivity of the infecting bacteria to select an antibiotic for local application.

Cruickshank and Lowbury (1952) consider that tissue culture overestimates the toxicity of antibiotics. For example, Jackson, Lowbury, and Topley (1951) found that penicillin cream used at 10,000 i.u./g. had no apparent ill effect on wound healing although the same concentration often produced necrotic skin cultures and reduced skin respiration by 36% after one day. Clearly an antibiotic applied to a surface wound will be continually diluted by exuded fluid and also removed from the site by the blood stream. Skin respiration experiments suggest that most of the antibiotics take time to exert a toxic action. It may be that a concentration of antibiotic, found toxic *in vitro*, when applied *in vivo* would have this concentration reduced to a safe level before it had time to exert its full toxic effect. It is possible that, if a concentration of antibiotic found toxic *in vitro* could be maintained *in vivo*, wound healing might be impaired. This point requires investigation.

I should like to thank Dr. J. P. Bull and Dr. E. J. L. Lowbury for much invaluable advice and criticism.

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THE DURATION OF ACTION OF SOME CARDIAC GLYCOSIDES AND AGLYCONES IN THE GUINEA-PIG

BY

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A method is described for determining the duration of action of cardiac glycosides and aglycones in the guinea-pig. It is based on their property of potentiating the cardiac response to adenosine. The method is particularly suitable for those drugs with a short duration of action, whereas previous methods are more suitable for those drugs with longer durations of action. The duration of action of one-fifth of the lethal dose has been found for: digoxigenin, lanatoside C, ouabain, digitoxigenin-3-one, digitoxin, 3-acetyldigitoxigenin, digoxin, digitoxigenin, lanatoside A; these drugs are arranged in order of increasing duration of action. The possible relationship between the elimination of these drugs and their duration of action can provide an estimate of their rates of elimination.

The determination of the elimination of cardiac glycosides by direct measurement of the amount of drug lost from the animal, or the amount remaining after certain periods of time, is not yet feasible. The existing methods are based on the persistence of effect of these drugs. In the method of Hatcher (1912, 1913), a known fraction of the lethal dose of the cardiac glycoside under examination is given and, after appropriate periods of time, the lethal dose of another (standard) cardiac glycoside is determined; the difference between this lethal dose and the usual lethal dose of the standard drug gives the equivalent dose as a fraction of the LD remaining effective in the animal after the elapsed period of time. Another method, introduced by Hauptstein (1927) and modified by Hoffman and Lendle (1951) depends on the differences in lethal doses observed with fast and slow rates of infusion of a cardiac glycoside. With these methods, large numbers of animals are required and estimates of precision are difficult to obtain.

Recently we proposed a method for the determination of the duration of action of cardiac glycosides which allows a value to be obtained from each animal used (Rand and Stafford, 1956). In this paper we describe the method in more detail and give results obtained with the following cardiac glycosides, ouabain, lanatosides A and C, digitoxin, and digoxin; with the aglycones digitoxigenin and digoxigenin, and the semi-synthetic derivatives, 3-acetyldigitoxigenin and digitoxigenin-3-one.

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METHODS

For the determination of the duration of action of cardiac glycosides, we used their property of potentiating the response to adenosine (Rand, Stafford, and Thorp, 1955). The guinea-pig under urethane anaesthesia (1.75 g./kg.) was given artificial respiration, the chest was opened, and a 1 mm. bore polythene cannula tied into the tip of the left atrium. Injections of adenosine were given through this cannula at 2 min. intervals. Adenosine (1 mg./ml.) was dissolved in 0.9% NaCl and 0.01 to 0.05 ml. was injected each time, either with an "Agla" micrometer syringe, or with a Palmer motor-driven syringe which delivered 0.5 ml./min. driven for 1 to 6 sec. With this latter device, pulses of the required duration from a photo-timer could be applied to the motor at 2 min. intervals. In either case, the dose of adenosine (10 to 50 μg.) was adjusted to produce heart block lasting for 3 to 5 sec. This response was measured from the electrocardiogram recorded with a Kelvin-Hughes ink writer. A number of injections of adenosine (6 to 10) were given to determine the mean initial response and its 95% confidence limits. The cardiac glycoside was then injected into a jugular vein and the regular injections of adenosine

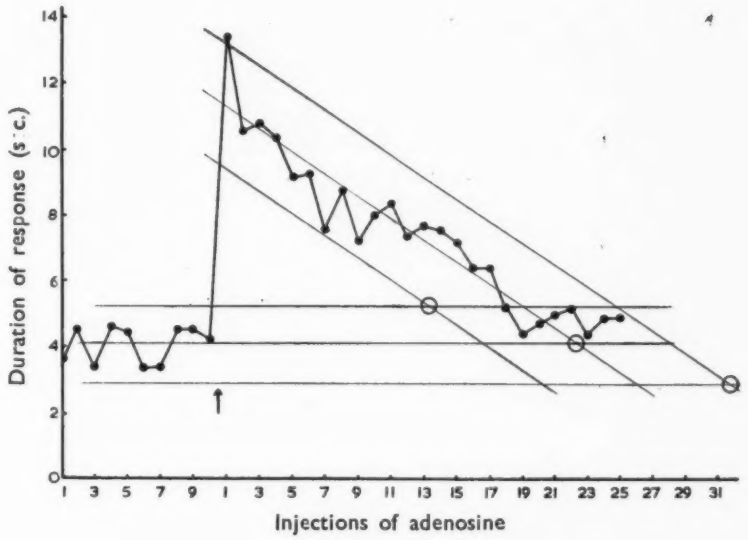

FIG. 1.—The ordinate gives the time in sec. during which heart block occurred in response to injections of adenosine (30 μg.) given at 2 min. intervals in the guinea-pig. There was a temporary potentiation of the response to adenosine after the injection of 1.28 mg./kg. of digitoxigenin-3-one at the arrow. The light horizontal lines are the mean and 95% confidence limits of the initial responses to adenosine. The light sloping lines are the regression and its 95% limits expressing the decrease in the potentiated response to adenosine with time.

continued. The response to adenosine was potentiated by the cardiac glycoside. The time taken until the potentiated responses to adenosine returned to their initial value has been taken as the duration of action of the dose of cardiac glycoside which had been given.

RESULTS

Constancy of the Response to Adenosine.—This method of determining the duration of action of cardiac glycosides depended upon the assumption that the guinea-pig would respond to successive injections of adenosine with constant periods of heart block for the duration of the experiment, that is, for 1 to 2 hr. The responses to injections of adenosine, given at 2 min. intervals, remained constant for 3 hr. in 5 experiments and for 6 hr. in one experiment. The constancy of the response was judged by the fact that the expected number of the responses were contained within the 95% confidence limits of the mean of the initial 10 responses.

The Determination of the Duration of Action.—The effect of a single dose of digitoxigenin-3-one on the responses to adenosine is shown in Fig. 1. In this experiment, the mean initial response was determined from 10 successive injections of adenosine. The mean time during which heart block occurred was 4 sec. with a standard deviation of 0.52 sec. The mean and the 95% confidence limits enclosing the results ($\pm$1.18 sec.) are shown as 3 horizontal lines.

Immediately following the injection of 1.28 mg./kg. of digitoxigenin-3-one, the response to adenosine was increased from a mean duration of 4 sec. up to 13.4 sec. The responses to successive doses of adenosine then decreased until (after 44 min.) they had the same duration as initially. To determine the point at which the potentiating action of digitoxigenin-3-one had worn off, we used the following procedure. The decay of the responses to adenosine was calculated as the regression of the response (duration) on time (in 2 min. intervals equal in number to the number of injections). This calculation was made beginning at the first response to adenosine showing the maximum potentiation up to the first response to adenosine which fell inside the 95% confidence limits of the mean initial values. In Fig. 1 the regression line for the decay of the responses to adenosine ($b = -0.332$) is drawn on the graph together with the lines representing the 95% confidence limits of the scatter about the regression line. The point chosen as the best estimate of the return of the potentiated responses to adenosine to their initial value is taken as the point of intersection of the extension of the mean initial responses with the extension of the regression line of decay. The greatest limits of this estimate are the extreme points of intersection of the lines of the 95% confidence limits. In Fig. 1, these points are represented by the open circles. In this experiment the effect of 1.28 mg./kg. of digitoxigenin-3-one persisted for 44 min. with a possible variation of 26 to 62 min. based on the extreme intersections of the lines enclosing the 95% confidence limits. The procedure described above has been carried out to provide an objective basis for judging the point of return

TABLE I

LETHAL DOSE OF SOME CARDIAC GLYCOSIDES AND AGLYCONES IN THE GUINEA-PIG

Drug	No. of Animals	Rate of Infusion (μg./kg./min.)	Lethal Dose (mg./kg.)	Standard Error
Lanatoside C	6	23– 41	0·679	0·038
Digoxin	7	21– 50	0·588	0·059
Digoxigenin	6	200– 300	6·80	0·83
Lanatoside A	5	62– 88	1·04	0·04
Digitoxin	6	50– 60	1·22	0·18
Digitoxigenin	7	150– 390	2·65	0·21
3-Acetyldigitoxigenin	7	300– 750	6·12	0·61
Digitoxigenin-3-one	4	580–1,100	6·41	0·70
Ouabain	6	11– 25	0·232	0·011

of the potentiated responses to their initial value and also to give an estimate of the precision of this point.

Lethal Doses of the Compounds Studied.—We have shown previously that the persistence of action of digoxigenin in the guinea-pig is proportional to the dose injected (Rand and Stafford, 1959). Therefore it is necessary to compare the durations of action of different cardiac glycosides and aglycones on some equitable basis. We have chosen to use one-fifth of the lethal dose of each compound. The lethal doses were determined in the guinea-pig under urethane anaesthesia with artificial respiration. A continuous infusion of the drug was given into a jugular vein until cardiac arrest, which was estimated from the electrocardiogram. The results are given in Table I.

Duration of Action.—The durations of action of one-fifth of the lethal doses of the cardiac glycosides and aglycones are shown in Table II. In each experiment, the procedure described above for digitoxigenin-3-one was carried out. In some experiments, the potentiated responses to adenosine did not decrease with time, or did so only slowly. In these, the regression line for decay was not significant and the result has been entered in Table II as greater than 2 hr. In all other experiments, the regression line for decay was highly significant.

DISCUSSION

Many previous workers have considered that the persistence of effect or duration of action of a cardiac glycoside-like drug is related to its rate of elimination from the animal (Lendle, 1936; Östling, 1947; Giertz, Hahn, and Schunk, 1954). If this is correct it is possible to present the results obtained on duration of action as rates of elimination of the various compounds studied as shown in Table III. This method of expressing

TABLE II

DURATION OF ACTION OF ONE-FIFTH OF THE LETHAL DOSE OF SOME CARDIAC GLYCOSIDES AND AGLYCONES

Drug	Dose (μg./kg.)	Duration of Action (min.)	95% Confidence Limits (min.)
Ouabain	46	42	39– 52
		40	32– 47
		37	15– 59
		37	20– 54
		27	17– 36
Lanatoside C	136	37	26– 48
		34	28– 40
		27	12– 42
		24	13– 34
		22	15– 28
Digoxin	118	>120	
		>120	
		>120	
		119	77–160
Digoxigenin	1,360	40	16– 47
		37	17– 63
		31	19– 45
		26	16– 36
		22	13– 28
		13	11– 16
Lanatoside A	208	>120	
		>120	
		>120	
		>120	
		>120	
		>120	
Digitoxin	244	>120	
		>120	
		113	50–126
Digitoxigenin	530	>120	
		>120	
		>120	
		>120	
		>120	
		>120	
		79	50–107
3-Acetyldigitoxigenin	1,224	>120	
		>120	
		140	57–214
		285	148–430
Digitoxigenin-3-one	1,282	84	34–135
		60	24– 94
		44	26– 62
		33	19– 46
		24	15– 34

TABLE III

MEAN RATES OF ELIMINATION OF SOME CARDIAC GLYCOSIDES AND AGLYCONES

Drug	No. of Expt.	Rate of Elimination		
		μg./kg./hr.	% LD/hr.	95% Limits
Ouabain	5	78·4	33·8	25·0–56·9
Lanatoside C	5	295	43·6	32·3–71·7
Digoxin	4	17·3	2·5	1·9– 3·9
Digoxigenin	6	3,387	43·9	36·7–79·0
Lanatoside A	6	0	0	
Digitoxin	3	55·7	4·5	3·2– 8·1
Digitoxigenin	7	57·7	2·2	1·6– 3·4
3-Acetyldigitoxigenin	4	197	3·2	2·1– 7·3
Digitoxigenin-3-one	5	1,909	29·7	20·4–54·5

the results allows comparisons to be made with other results recorded in the literature on the rates of elimination of these drugs in which the actual measurement made was on persistence of the effect.

The average rate of elimination of ouabain was found to be 34% of the lethal dose/hr., or 78.4 μg./kg./hr. The rates of elimination of ouabain given by Hoffman and Lendle (1951) were 68 to 180 μg./kg./hr., and their mean value was 35% LD/hr. Calculations made from the results of Herre (1937) gave a rate of elimination of ouabain of 92 μg./kg./hr., which was 30% of the lethal dose/hr. as measured by the same author.

Herre (1937) showed that digitoxin was not eliminated by the guinea-pig, though more recently Hoffman and Lendle (1951) found that digitoxin was eliminated by the guinea-pig at a rate of 20 to 29% of the lethal dose/hr. We have found that digitoxin was only eliminated in 1 out of 3 experiments, at a rate of 13.6% of the lethal dose/hr.

Hoffman and Lendle (1951), who used the method of Hauptstein (1927), estimated that the rate of elimination of digitoxigenin in the guinea-pig is 1.0 to 1.5 mg./kg./hr. However, we were unable to demonstrate any appreciable elimination of digitoxigenin by the guinea-pig, the response to adenosine remaining potentiated for at least 2 hr. following the injection of digitoxigenin in 6 out of 7 animals tested. When we determined the toxicity of digitoxigenin in the guinea-pig, we found that, unless it was infused rapidly (>150 μg./kg./min.) so that cardiac arrest was produced within approximately 15 min., it was impossible to attain a lethal dose. With slower rates of infusion an ectopic ventricular rhythm was produced which persisted until enormous doses of digitoxigenin had been injected (up to 20 mg./kg.) and the experiments were frequently terminated without death of the animal. These observations suggest that digitoxigenin is eliminated very rapidly in the guinea-pig. In order to explain the discrepancy between our results using the effect of digitoxigenin on the response to adenosine and the results of Hoffman and Lendle (1951) which are in accord with our observations made during the determination of the lethal dose of digitoxigenin, we offer the following explanation. The slope of the line relating dose to rate of elimination may be considerably steeper for digitoxigenin than was shown for digoxigenin (Rand and Stafford, 1959). This would mean that although no appreciable elimination of digitoxigenin could be shown with the doses which we used (20% of LD), with the higher concentrations of digitoxigenin which would be present if rate of elimination were measured by the method of Hauptstein (1927), a more rapid rate of elimination would be observed. We have not yet, however, tested this possibility experimentally. Another possibility is that the direct toxic effect of digitoxigenin itself on the heart which Hoffman and Lendle (1951) measured is transient, whereas the indirectly observed action of potentiating the response to adenosine is longer lasting.

Our results give no information about the location of the cardiac glycosides or aglycones or their cardio-active metabolites which are potentiating the responses to adenosine. It is unlikely that potentiation of the response to adenosine would be proportional to the concentration of cardiac glycoside in the blood, as there are observations in many species which indicate that cardiac glycosides are removed from the blood within a few minutes after injection (Hatcher and Eggleston, 1919; Hanzlik and Wood, 1929; and others mentioned by Okita, Talso, Curry, Smith, and Geiling, 1955). It is thought that this is due to their being taken up by various body tissues, particularly by the liver. The potientiated response of the heart to adenosine may reflect the concentration of drug in the heart muscle.

There has been much work concerning the "binding" of cardiac glycosides to the heart muscle of various species. Recent experiments with radioactive drugs indicate that the heart takes up cardiac glycosides, although to a lesser extent/g. of tissue than many other organs (Okita *et al.*, 1955). It may be that only the fraction of the drug actually fixed in the heart muscle is responsible for the potentiation of the response of the heart to adenosine, in which case the concept of elimination of the drug as an explanation for the decrease in effect with time after injection has the more limited sense of elimination from the heart.

The unexpected resistance of digitoxigenin to elimination has already been considered. Acetylation of the 3-OH group of digitoxigenin reduces its toxicity by 60%, but acetyldigitoxigenin is only eliminated slightly more rapidly than digitoxigenin. However, digitoxigenin-3-one, with approximately the same cardiac activity as 3-acetyldigitoxigenin, acts much more briefly. This would suggest that the 3-OH group is a point of attachment to receptor sites for pharmacological activity, and that either acetylation or oxidation of the 3-OH group weakens this

attachment, which is reflected in the lower toxicity of these compounds, and places them in a position where they are more likely to be lost from the heart. However, acetylation of the 3-OH only slightly increases the rate of elimination, whereas oxidation greatly facilitates it. This may be because oxidation of the 3-OH is one of the stages in the detoxication of the aglycones.

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EFFECT OF HISTAMINE ON GANGLIONIC TRANSMISSION

BY

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Histamine has been shown to depress transmission through the perfused superior cervical ganglion of the cat when doses of 150 μg. or more were administered. The intensity of the ganglionic block was related to the dose of histamine employed. In one-third of the experiments, a slow contracture of the nictitating membrane occurred after histamine had been injected; the contracture lasted up to 10 min., and subsequent injections of histamine gave rise to progressively smaller responses. The blocking action of histamine was evident in all experiments and was the most prominent feature observed. Histamine in a sub-depressant dose enhanced the action of the competitive blocking agents tetraethylammonium and hexamethonium, and also the depolarizing blocking agents tetramethylammonium and nicotine. The possible physiological rôle of histamine in the autonomic nervous system is discussed.

Although the presence of histamine in nervous tissue has been reported by a number of authors, its physiological significance remains obscure. Harris, Jacobsohn, and Kahlson (1952) demonstrated the presence of histamine in considerable quantities in the region of the hypothalamus, and von Euler and Astrom (1948), von Euler (1949), and Werle and Weicken (1949) showed that certain peripheral nerve fibres, particularly the sympathetic postganglionic fibres, were rich in histamine. The release of histamine from certain peripheral nerves on electrical stimulation was reported by Kwiatkowski (1943); and Gertner (1955) has shown that the histamine liberators 48/80 and propamidine depress transmission through the perfused superior cervical ganglion of the cat.

The effects of exogenous histamine on transmission in the nervous system would appear to require further study since divergent results have appeared in the literature. Feldberg and Vartianen (1935) were unable to demonstrate any action of histamine on transmission through the sympathetic ganglion, whereas Burn and Trendelenburg (1954), Trendelenburg (1954, 1955), and Konzett (1952) described a stimulant action of histamine. The present investigation was therefore undertaken in an attempt to establish the actions of histamine on transmission through the sympathetic ganglion and also to ascertain its effects on the actions of ganglion blocking agents.

METHOD AND MATERIALS

Cats of either sex weighing 2 to 3.5 kg. were anaesthetized with pentobarbitone sodium 30 mg./kg. The superior cervical ganglion was then isolated and perfused *in vivo* according to the method of Feldberg and Gaddum (1934) and with the later modification of MacIntosh, as reported by Perry (1953). The perfusion fluid was Locke solution made up as follows: NaCl, 9.2 g.; KCl, 0.42 g.; $CaCl_2$, 0.24 g.; $NaHCO_3$, 0.15 g.; glucose, 1 g./l. of solution. 25 cats were employed.

The nictitating membrane was connected to a frontal writing lever and the response recorded on a smoked paper. The preganglionic cervical sympathetic trunk was stimulated for 10 sec. in each minute by rectangular pulses from a Grass stimulator at 10/sec., of 0.5 m.sec. duration and supramaximal voltage usually 10 V.

The drugs used were dissolved in Locke solution and injected into the perfusion stream in a volume never exceeding 0.3 ml. Histamine was used as the acid phosphate unless otherwise indicated and the doses are quoted as histamine base. The doses of all other drugs are given in terms of their salts.

RESULTS

Effect of Histamine on Ganglionic Transmission

Graded doses of histamine were injected into the perfusion stream. Quantities of histamine from 1 to 100 μg. produced little noticeable effect, whereas larger amounts (100 to 150 μg.) produced a slight depression of transmission. With doses of 300 μg. of histamine, a greater degree of

depression was evident in all the preparations tested and doses of 600 μg. produced a profound inhibition of ganglionic transmission. In two ganglia, there was a complete block of transmission with 600 μg. of histamine lasting for 10 min. Recovery was complete within a period of 20 min. Fig. 1 illustrates the effects of graded doses of histamine.

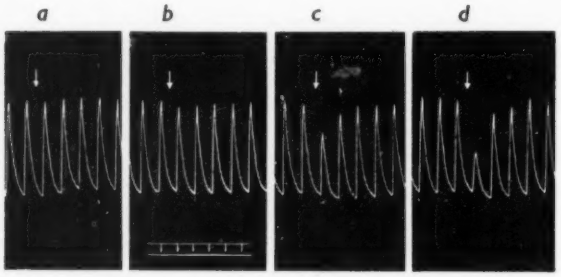

FIG. 1.—Graded responses to graded doses of histamine. Cat 2.5 kg., pentobarbitone anaesthesia, perfused superior cervical ganglion preparation. Contractions of the nictitating membrane in response to electrical stimulation of the preganglionic cervical sympathetic trunk for 10 sec. every min. Injections of histamine into the ganglion perfusion fluid at the arrows (*a*) 75 μg.; (*b*) 150 μg; (*c*) 300 μg., and (*d*) 600 μg. Time, 1 min.

The possibility that this block of transmission was due to the action of the phosphate anion of the histamine molecule was then studied. Histamine dihydrochloride containing the same proportion of base as the phosphate salt produced identical degrees of ganglionic block. This indicated that the depression of transmission was due to the histamine *per se* and not to the anion portion of the molecule. Since the acid phosphate of histamine has a *p*H of 4.2, a control was carried out by preparing a buffer solution of M/15 Na_2HPO_4 and NaH_2PO_4 of identical *p*H and its effect studied on transmission. When 0.2 ml. of this buffered solution was injected into the perfusion stream, no noticeable effect on transmission was observed. It was thus evident that histamine itself, in the doses employed, depressed ganglionic transmission.

In one-third of the experiments it was noted that the first injection of histamine (usually 150 μg.) produced a slow contracture of the nictitating membrane, often sustained for up to 10 min. This occurred in the absence of electrical stimulation of the ganglion (Fig. 2). When the identical dose of histamine was given after recovery, the stimulant effect of the second dose was greatly diminished. A third injection given after recovery produced a still smaller contraction of the nictitating membrane. Both the chloride and the phosphate salts of histamine also showed this tachyphylactic stimulant effect, which is thus clearly due to histamine itself.

This slow contracting action of histamine was completely independent of the other and much more prominent action of histamine, namely that of depressing transmission. Histamine depressed transmission in all the preparations tested and there was no tachyphylaxis in respect of this depression.

Effect of Histamine in the Presence of Ganglion Blocking Agents

Doses of ganglion blocking agents were chosen, which, when injected into the perfusion stream, produced about 30% to 50% depression of transmission. This dose was given three times to determine whether potentiation occurred on repeated injections of the blocking agent. A test dose of histamine was selected which had negligible effects on transmission. The ganglion blocking agent was injected into the perfusion stream and followed within a minute by the test dose of histamine. Two representative competitive blocking agents, tetraethylammonium and hexamethonium, and two depolarizing blocking agents, tetramethylammonium and nicotine, were employed.

Hexamethonium Chloride

Usually doses of 10 μg. of hexamethonium produced approximately a 50% block of transmission. When the test dose of 150 μg. of histamine was injected immediately after the

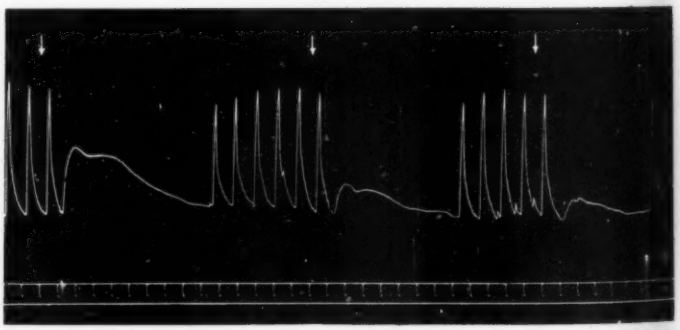

FIG. 2.—Slow contraction produced by histamine. Cat 3.0 kg., pentobarbitone anaesthesia, perfused superior cervical ganglion preparation. Contractions of the nictitating membrane to electrical stimulation of the preganglionic cervical sympathetic trunk for 10 sec. every min. Injections of 150 μg. of histamine into the perfusion fluid at (*a*), (*b*), and (*c*). Electrical stimulation was discontinued 1 min. after the injection of histamine and started again when recovery was complete. Time, 1 min.

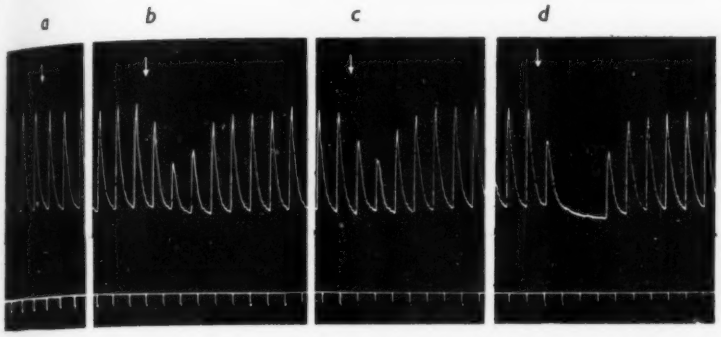

FIG. 3.—Enhancement of the action of hexamethonium by histamine. Cat 2.8 kg., pentobarbitone anaesthesia, perfused superior cervical ganglion preparation. Contractions of the nictitating membrane to electrical stimulation of the cervical sympathetic trunk for 10 sec. every min. At (*a*) 150 μg. of histamine; at (*b*) and (*c*) 10 μg. of hexamethonium chloride; at (*d*) 10 μg. of hexamethonium followed immediately by 150 μg. of histamine. Complete ganglion block arose. Time, 1 min.

third dose of hexamethonium, the degree of depression of transmission through the ganglion was increased both in intensity and duration (Fig. 3). This potentiation by histamine of the inhibiting action of hexamethonium could be repeated after re-establishment of normal transmission through the ganglion.

Tetraethylammonium Chloride

Tetraethylammonium chloride in a dose of about 100 μg. produced about a 50% block of transmission through the ganglion. The procedure as outlined for hexamethonium was followed, and similar results obtained. The response of the ganglion to the tetraethylammonium was greatly enhanced by histamine and this could be repeated on re-establishment of normal transmission 10 to 15 min. following the last injection of histamine.

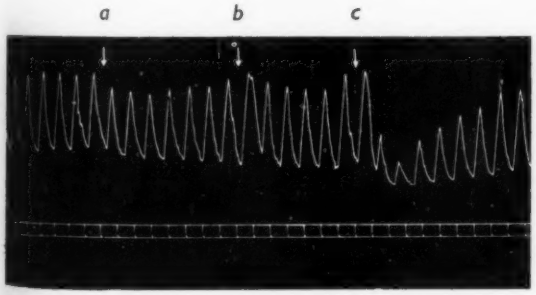

FIG. 4.—Enhancement of the depressant action of tetramethylammonium by histamine. Cat 3.0 kg., pentobarbitone anaesthesia, perfused superior cervical ganglion preparation. Contractions of the nictitating membrane to electrical stimulation of the cervical sympathetic trunk for 10 sec. every min. Injections into the fluid perfusing the ganglion at (*a*) 150 μg. of histamine; at (*b*) 10 μg. of tetramethylammonium bromide; at (*c*) 10 μg. of tetramethylammonium bromide followed immediately by 150 μg. of histamine. Time, 1 min.

Tetramethylammonium Bromide.—A dose of tetramethylammonium bromide, usually 10 μg., was chosen which initially stimulated the ganglion cells and then slightly depressed transmission. When histamine was given after tetramethylammonium, a greatly enhanced depression of transmission was observed (Fig. 4). In all experiments only the post-stimulatory depression phase of the action of tetramethylammonium was potentiated and not the initial stimulant phase. In general, these results could be repeated after complete recovery (10 to 15 min.) with no diminution of effect.

Nicotine

A dose of 5 μg. of nicotine hydrogen tartrate usually stimulated the ganglion cells initially but subsequently depressed transmission to approximately 50% of the original value. After the injection of nicotine, histamine caused considerable enhancement of the depression of transmission (Fig. 5), but only the post-stimulatory depression of transmission was potentiated and not the initial stimulant phase. An injection of 5 μg. nicotine given when recovery was complete (18 min. later) showed that the potentiating action of histamine had entirely disappeared.

DISCUSSION

It is clear from the experiments reported here that histamine *per se* has the ability to depress transmission when injected into the perfused superior cervical ganglion of the cat. This was a distinct pharmacological effect as is evident from the dose/response relationships. The reports in the literature concerning the lack of activity of histamine on the ganglion may be due to its use in insufficient doses.

The stimulating action of histamine on ganglia, which has been reported by other investigators, may be related to the slow contraction described in this paper. This phenomenon, which exhibits tachyphylaxis, is of considerable interest. No explanation as to why it occurred in only one-third of the experiments readily presents itself. The possibility exists that, in a small number of ganglia, some sympathomimetic amine may be released by histamine from chromaffin tissue present in the ganglion and that this may slowly diffuse out and produce the contraction

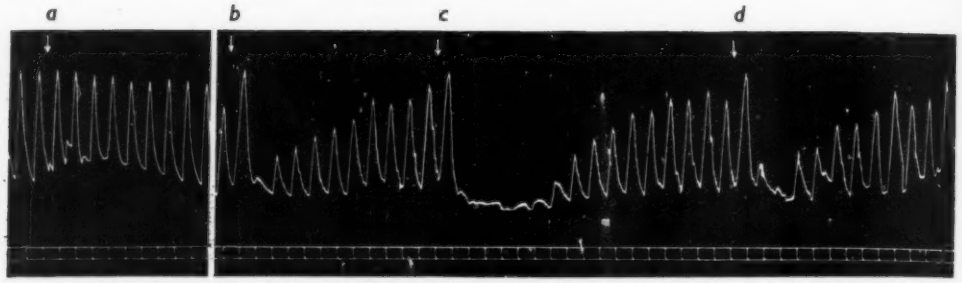

FIG. 5.—Enhancement of the depressant action of nicotine by histamine. Cat 2.9 kg., pentobarbitone anaesthesia, perfused superior cervical ganglion preparation. Contractions of the nictitating membrane to electrical stimulation of the cervical sympathetic trunk for 10 sec. every min. At (*a*) 150 μg. of histamine was injected; (*b*) 5 μg. of nicotine hydrogen tartrate; (*c*) 5 μg. of nicotine hydrogen tartrate followed immediately by 150 μg. of histamine; and (*d*) 5 μg. of nicotine hydrogen tartrate. Time, 1 min.

of the nictitating membrane. A point in favour of such a hypothesis is that it is a tachyphylactic phenomenon suggesting that the substance responsible may become exhausted. The fact that this slow contraction is observed would explain why histamine has been reported by some to exert a stimulant action on ganglia, masking the more important and prominent action of histamine in depressing transmission.

The doses of histamine injected into the ganglion may appear to be somewhat larger than ordinarily required to obtain a physiological response such as that on the cardiovascular system. It is important, however, to point out that, although this preparation remains *in situ*, it is isolated from the normal metabolites of the body as well as from the blood stream. In addition, and possibly of greater importance, is the lack of any substantial evidence on the ability of nervous tissue to metabolize histamine, hence it cannot be concluded justifiably at this stage that the doses employed in our experiments were excessive.

At present it is not possible to claim any physiological rôle for histamine in autonomic transmission, but it is of interest that there may be an analogy between histamine and acetylcholine in this situation. It should be recalled that the dose of acetylcholine required to stimulate the ganglion is at least 1,000 times greater than the amount released on nerve stimulation even in the presence of an anticholinesterase. Thus the dosage relationships of histamine may not be unlike those of acetylcholine.

The action of histamine in potentiating the effects of the ganglion blocking agents has its analogy in the results reported by Schenk and Anderson (1958) that histamine potentiates the action of certain of the neuromuscular blocking agents at the neuromuscular junction. These authors, however, observed a difference in action of histamine on the competitive as compared to the depolarizing neuromuscular blocking agents. Bovet-Nitti, Kohn, Marotta, and Silvestrini (unpublished observations), however, found that histamine potentiated both the competitive and depolarizing neuromuscular blocking agents *in vivo* in the rabbit and rat using the gastrocnemius and masseter muscle preparations. The experiments reported here for the ganglionic synapse appear to be more consistent with these latter findings, for we have found a similarity of action of histamine in enhancing both the competitive and the depolarizing ganglionic blocking agents. The physiological significance of these findings remains obscure.

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THE RELATION BETWEEN TEMPERATURE AND CHANGES IN ION CONCENTRATION ON VENTRICULAR FIBRILLATION INDUCED ELECTRICALLY

BY

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Further observations have been made on ventricular fibrillation induced by electrical stimulation in the isolated rabbit heart, in extension of the finding that fibrillation was arrested by cooling from 37° to 32°. The observations now made suggest that at 37° the isolated rabbit heart perfused with Locke solution is on the edge of anoxia, but that at 32° the oxygen supply is adequate. At the lower temperature the proportion of hearts fibrillating at any given potassium concentration was reduced, so, although the proportion of hearts fibrillating rose as the potassium concentration fell, even when the latter was one-quarter of the normal the proportion was not higher than 67%. The relation of the concentration of calcium ions to the proportion of hearts fibrillating changed from biphasic at 37° to almost rectilinear at 32°, the proportion increasing as the concentration of calcium ions rose. It was confirmed that a reduction in sodium concentration also raises the proportion of hearts fibrillating.

Ventricular fibrillation can be produced in the Langendorff preparation of the isolated rabbit heart by electrical stimulation of the ventricles. When the stimulation is stopped the heart either reverts to normal rhythm or fibrillation continues. Armitage, Burn, and Gunning (1957) investigated various factors which determined whether it continued, among them the amount of potassium and of calcium ions in the perfusing solution. They found, for example, that the proportion of hearts which continued to fibrillate rose as the amount of potassium was reduced. Kärki (1958) made a more complete study of changes in ion concentration, and Goodford (1958) investigated the action of metabolic inhibitors. He found that metabolic inhibitors caused fibrillation to persist and also that a lack of dextrose would precipitate fibrillation. In the course of his work he observed that, when a heart was in continuous fibrillation at 37°, the fibrillation was arrested on cooling to 32°. At that temperature, electrical stimulation did not induce fibrillation, though when the temperature was raised to 37° it did so once more. Goodford suggested that this effect might depend on the relation between the amount of oxygen available and the amount required by the heart. The oxygen available was the amount in solution, which was much less than the amount in blood. Since the oxygen requirement was greater at 37° than at 32° it might be that the occurrence of fibrillation at the higher temperature was due to an insufficient supply of oxygen at that temperature. Kärki, for example, found that at 37° no less than 15 out of 28 hearts fibrillated continuously after electrical stimulation although the fluid perfusing them was of the usual ionic concentration.

These observations suggested that a clearer picture of the effect of changes in ionic concentration would be obtained if experiments were made at 32°. Such experiments are now described.

METHOD

The method used was similar to that described by Armitage *et al.* (1957). The hearts, which were freshly excised from rabbits, were perfused through the coronary vessels by the method of Langendorff. The temperature of the fluid perfusing the heart was maintained constant despite variations of coronary flow by using the device of Saxby (1956).

The usual perfusion fluid described by McEwen (1956) contained NaCl 132 mM, KCl 5.6 mM, $CaCl_2$ 2.2 mM, NaH_2PO_4 0.92 mM, $NaHCO_3$ 2.5 mM, dextrose 11.1 mM, sucrose 13.1 mM. The solution was saturated with O_2 95% + CO_2 5%. The pressure head at which the hearts were perfused was 54 cm. water.

The ventricles were stimulated by platinum electrodes, one piercing the wall of the left ventricle at the apex, and the other near the base midway between the origins of the right and left coronary arteries. When stimulation was not being applied, the electrodes were used as leads to an electrocardiograph (Cossor model 1314). A mechanical record was obtained on a smoked drum by a thread attached to the electrode in the apex of the left ventricle. The stimuli were rectangular current pulses of 0.75 msec. duration and approximately 1 to 2 mA. Stimulation was begun at a rate of about 580/min., and the rate was increased until fibrillation was observed, the maximum rate being 1,200/min. The hearts were stimulated for 5 min.

When stimulation stopped each heart was observed to see if it reverted to a normal rhythm. Hearts reverting within 15 min. were recorded as "not fibrillating"; hearts not reverting within this time were recorded as "fibrillating."

The hearts were first perfused for 30 min. with the normal solution; this was then changed to the solution under test, and at the end of a further 30 min. stimulation was applied.

Results

Variations in Potassium Concentration

Observations were made at the usual potassium concentration, 5.6 mM, and at concentrations one-half and one-quarter of this. The results are given in Table I together with the results obtained at 37° by Kärki. The general trend was the same at 32° as at 37°, the proportion of hearts which fibrillated increasing as the potassium concentration fell. At each concentration, however, the proportion fibrillating was much less at 32° than at 37°. The difference was particularly noticeable when the potassium concentration was 1.4 mM because at 37° many hearts fibrillated spontaneously without stimulation, while at 32° only 6 out of 9 fibrillated when stimulated.

TABLE I

PROPORTION OF HEARTS FIBRILLATING AT DIFFERENT POTASSIUM CONCENTRATIONS, WHEN PERFUSED AT 32° OR 37°

Ion Conc. mM	37°		32°	
	Proportion	%	Proportion	%
11·2	0/8	0		
5·6	15/28	54	2/8	25
2·8	12/13	92	3/9	33
1·4	10/10	100	6/9	67

Six hearts were examined at 27°; at this temperature, even when the potassium concentration was only 1.4 mM, none fibrillated. The relation between potassium concentration and the proportion of hearts fibrillating is shown in Fig. 1 (curve **A**).

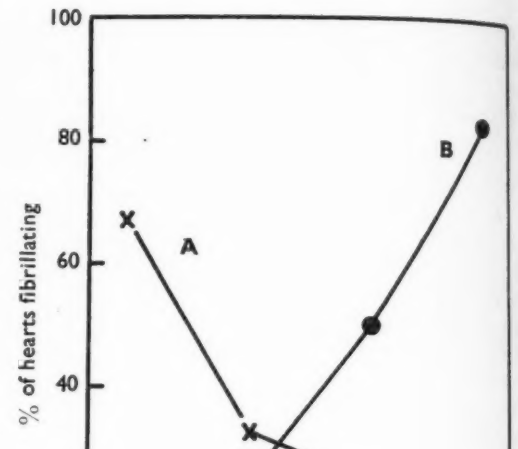

FIG. 1.—Percentage of hearts fibrillating at 32°. **A.** Calcium 2·2 mM and different potassium concentrations. **B.** Potassium 5·6 mM and different calcium concentrations.

Variations in Concentration of Calcium Ions

When the potassium concentration was kept at 5.6 mM and the concentration of calcium ions was varied, the proportion of hearts fibrillating varied with the calcium ion concentration, rising to 83% when this was increased fourfold and falling to 16% at half the usual concentration (see Fig. 1, curve **B**, and Table II).

TABLE II

PROPORTION OF HEARTS FIBRILLATING AT DIFFERENT CALCIUM ION CONCENTRATIONS, WHEN PERFUSED AT 32° OR 37°

Ion Conc. mM	37°		32°	
	Proportion	%	Proportion	%
8·8	—	—	5/6	83
4·4	12/12	100	3/6	50
2·2	15/28	54	2/8	25
1·1	7/15	47	1/6	16
0·55	3/8	38	—	—
0·275	6/21	29	—	—

Variations in Sodium Concentration

When the concentrations of potassium and calcium ions were kept normal, and the sodium concentration was reduced to one-half, the percentage of hearts fibrillating at 37° rose from

54 to 100. In experiments at 32°, when a similar reduction in sodium concentration was made, the tonicity being maintained by adding sucrose, 4 out of 6 hearts fibrillated, the percentage rising from 25 to 67.

Effect of Changes in Ionic Concentration on Normal Cardiac Rhythm

Observations were also made on the amplitude and rate of beat of the heart. At 32° the amplitude was larger and an increase in rate was often observed when either the potassium or the sodium concentration was reduced, and also when the calcium ion concentration was increased.

At 37°, when hearts were perfused with 1.4 mM potassium, it was usually observed that the amplitude increased steadily and the rate slightly until eventually there was spontaneous fibrillation. At 32°, in 5.6 mM potassium, the beats were small and irregular, but on changing to 1.4 mM potassium they became large and regular.

It was also noticed that it was more difficult to cause a heart to fibrillate by electrical stimulation at 32° than at 37°. This was especially noticeable in 1.1 mM calcium. Currents as large as 10 mA. could be used with some hearts without causing fibrillation.

Discussion

The results obtained give general support to the conclusions already reached by Kärki (1958) that a reduction of potassium and of sodium favours fibrillation while a decrease of calcium ion makes fibrillation less likely. The curve relating the proportion of hearts fibrillating to the calcium concentration which Kärki (1958) obtained, though free from the two peaks described by Armitage *et al.*, was of curious shape, and indicated the influence of more than one factor. The present observations at 32° have not this complexity, and they show that the effect of varying the concentration of calcium ions affects the proportion of hearts fibrillating in a roughly rectilinear manner. This is a more probable result.

The observation that hearts could not be made to fibrillate at 27°, even when the potassium concentration was reduced to 1.4 mM, is in agreement with the results recently published by Covino and Beavers (1958), who found that rabbit hearts would not fibrillate at temperatures below 27°. They considered that this depended on the species, since fibrillation occurred at low temperatures in the dog.

The observations made by Kärki (1958) on the effect of changes in potassium, calcium, and sodium concentration seem to have been complicated by the fact that at 37° the isolated rabbit heart perfused with Locke solution very often suffers from an insufficient oxygen supply. The conditions which favour fibrillation (low potassium and sodium and high calcium concentrations) are generally accompanied by an increase in amplitude of the heart beat. Thus it appears likely that this increased work done by the heart, which would require extra oxygen, contributes to the spontaneous fibrillation which often occurs at 37° when the potassium concentration is low. That the isolated rabbit heart perfused at 37° may be on the border of anoxia is not generally known, and, while it has long been the practice to study isolated atria at 29°, it has been customary to perfuse the whole heart at 37°. The evidence now described suggests that the temperature should not be higher than 32°.

I would like to express my thanks to Professor J. H. Burn for suggesting this work to me. The electrocardiograph was very kindly supplied to the Department by the Wellcome Trust.

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OXIMES OF αω-DIQUATERNARY ALKANE SALTS AS ANTIDOTES TO ORGANOPHOSPHATE ANTICHOLINESTERASES

BY

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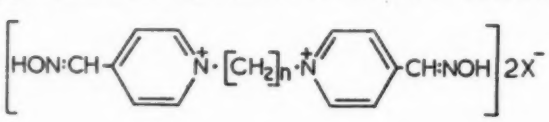
Sixteen compounds of the general structure $\{HON:CH.C_5H_4N^+.[CH_2]_n.R^+\}2Br^-$ have been synthesized in which the position of the oxime group in the pyridine ring, the second charged group R^+ and the number of methylene groups between the charged atoms have been varied. The rate at which these compounds reactivate cholinesterase inhibited by ethyl pyrophosphate has been studied and a number have been found which are more active than 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate. Since considerable variation in structure was found among those compounds which are better reactivators than the latter, the concept that 2-hydroxyiminomethyl-*N*-methylpyridinium salts are unique in their ability to fit the surface of the inhibited enzyme is no longer tenable. The reactivating power of these oximes correlated well with their ability, when given in conjunction with atropine, to save the lives of mice poisoned by ethyl pyrophosphate. The most effective compounds, *NN'*-trimethylenebis-(4-hydroxyiminomethylpyridinium bromide) and *NN'*-hexamethylenebis(2-hydroxyiminomethylpyridinium bromide), contained a further oxime group in R^+, but the second oxime group was not essential for high activity. These new oximes were also superior in saving the lives of mice poisoned with sarin (isopropyl methylphosphonofluoridate), but the improvement was not as dramatic as when the mice were poisoned with ethyl pyrophosphate. The toxicity of the compounds varied with both *n* and R^+ and was unrelated to the therapeutic potency.

Oximes derived from pyridinium aldehydes have been shown to be potent reactivating agents for cholinesterase inhibited by organophosphates and of considerable value in treating organophosphate-poisoned animals, especially when given in conjunction with atropine. Until recently, the most effective compound in both these respects was 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (pyridine-2-aldoxime methiodide; P2AM) or the methanesulphonate (P2S) (for review see Davies and Green, 1959). Poziomek, Hackley and Steinberg (1958) and, independently, Hobbiger, O'Sullivan and Sadler (1958) have described a new group of oximes, *NN'* - polymethylenebis(4 - hydroxyiminomethyl - pyridinium) compounds, with general structure (I) which were reported to be at least as effective,



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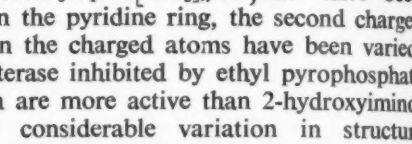

II

or even better, both as reactivators and as antidotes to organophosphates. We have examined sixteen compounds with the more general structure (II), in which we have varied the number of hydroxyiminomethyl groups and their positions in the pyridine ring or rings, the number of carbon atoms between the two charged atoms, and the nature of the second charged group R^+. These compounds have been tested for their ability to inhibit cholinesterase and to reactivate cholinesterase inhibited with organophosphate, for their ability to save the lives of poisoned mice, and for their intrinsic toxicity. A moderately good relationship has been shown between life-saving properties and ability to reactivate, whereas none can be seen between either of these properties and intrinsic toxicity.

METHODS

Preparation of Oximes. — 2-Hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate was prepared as described by Davies, Green, and Willey (1959). Symmetrical compounds of the formula (I), but in which the position of the hydroxyiminomethyl groups in the rings was varied, were obtained by the method of Poziomek *et al.* (1958) from the appropriate pyridine aldoxime. For compounds with the hydroxyiminomethyl group in the 2-position in the pyridine ring, acetone was preferred as a solvent to ethanol, but even so yields were only about 1 to 2%. Unsymmetrical compounds (II) in which R+ was a charged group such as pyridinium, isoquinolinium, or triethylammonium, were prepared by either of two routes. Yields by both methods were generally satisfactory.

(i) The tertiary nitrogen compound was boiled under reflux with 4 mol. equivalents of the polymethylene dibromide in anhydrous benzene. The *N*-ω-bromoalkyl compound crystallized out on cooling. This compound (in some cases after recrystallization from ethanol) was dissolved in anhydrous ethanol and was boiled under reflux for 16 hr. with 1 mol. equivalent of 4-hydroxyiminomethylpyridine. Sometimes the diquaternary oxime crystallized out on cooling, but in some cases, especially when the second base was aliphatic, the product was obtained on evaporation of the solvent as a deliquescent gum which solidified only after prolonged drying over P_2O_5.

(ii) During the preparation of the symmetrical dioximes, some *N*-(ω-haloalkyl)-hydroxyiminomethylpyridinium halide was often obtained as a by-product. These compounds, when boiled in ethanol with an unsaturated heterocyclic base, gave the desired unsymmetrical diquaternary oximes.

Table I shows the compounds prepared, and their elemental analyses and melting points. For ease of reference the structures have been indicated by the following code: The position of the hydroxyiminomethyl group in the pyridine ring is indicated by the first figure and, in the case of dioximes, also by the third figure. The number of methylene groups between the quaternary atoms is indicated by the second figure. In unsymmetrical compounds the nature of the group R+ (formula II) is indicated by a terminal letter or letters. For example, *NN'*-hexamethylenebis(4 - hydroxyiminomethylpyridinium bromide) is designated C4.6.4, and trimethylene-1-(3-hydroxyiminomethylpyridinium) - 3 - isoquinolinium dibromide is designated C3.3.IQ. The initial letter C denotes this particular series of synthetic compounds.

Inhibition of Cholinesterase by the Oximes.— Washed human erythrocytes suspended in an equal volume of 0.9% NaCl were used as the source of true cholinesterase. Activity of cholinesterase in the presence of most of the compounds was determined by continuous titration at *p*H 7.4 and 25°. The acid produced when 0.5 ml. of the enzyme preparation was added to a stirred mixture of 4.4 ml. of 0.3M-KCl, 0.5 ml. of 0.1M-acetylcholine iodide, and 0.6 ml. of oxime dissolved in 0.1M-KCl was titrated with 0.1N-NaOH.

Reactivation of Cholinesterase.—Washed human erythrocytes were shaken gently for 30 min. at 25° with an equal volume of 0.9% NaCl containing 10^{-6}M ethyl pyrophosphate. The cells were washed with ice-cold saline to remove excess ethyl pyrophosphate and were then suspended in an equal volume of saline and stored in a refrigerator until used. 10 ml. of the cell suspension was mixed with 10 ml. of a solution of reactivator in barbitone buffer *p*H 7.4 (0.01M barbitone sodium, 0.002M-KH_2PO_4 and 0.3M-KCl) kept at 25°. At suitable times, 1 ml. samples were withdrawn and their cholinesterase activity determined by the electrometric method

TABLE I

COMPOUNDS PREPARED

Code numbers, empirical formulae, analyses, and melting points of the oximes tested. Del.=deliquescent; ST, *S*-thiouronium; P, pyridinium; MP, 4-methylpyridinium; NEt_3, triethylammonium; IQ, isoquinolinium. For explanation of code see text.

Code No.	Empirical Formula	Analysis						M.P. °C.
		C		H		N		
		Calc.	Found	Calc.	Found	Calc.	Found	
C4.3.ST	$C_{10}H_{16}ON_4SBr_2$	30·1	30·1	3·8	4·3			205–6
C4.2.4	$C_{14}H_{16}O_2N_4Br_2$					13·0	12·8	285
C4.3.P	$C_{14}H_{17}ON_3Br_2$					10·4	10·3	212
C2.3.2	$C_{15}H_{16}O_2N_4Br_2$	40·4	40·0	4·1	4·4			226–8
C3.3.3	$C_{15}H_{18}O_2N_4Br_2$	40·4	39·9	4·1	4·4			203
C4.3.4	$C_{15}H_{18}O_2N_4Br_2$	40·4	40·3	4·1	4·3	12·6	12·1	222
C4.3.MP	$C_{15}H_{19}ON_3Br_2$					10·1	9·8	205–6
C4.3.NEt_3	$C_{15}H_{27}ON_3Br_2$	42·4	42·1	6·4	7·0			Del.
C4.4.4	$C_{16}H_{20}O_2N_4Br_2$	41·7	41·1	4·4	4·9			245–6
C3.3.IQ	$C_{18}H_{19}ON_3Br_2$	47·7	47·5	4·2	4·7	9·3	8·7	Del.
C4.3.IQ	$C_{18}H_{19}ON_3Br_2$					9·3	9·1	215–6
C2.3.IQ	$C_{18}H_{19}ON_3I_2$					7·7	7·4	219
C2.6.2	$C_{18}H_{24}O_2N_4Br_2$	44·3	43·5	5·0	5·3	11·5	11·0	205–7
C3.6.3	$C_{19}H_{24}O_2N_4Br_2$	44·3	44·3	5·0	5·3			240
C4.6.4	$C_{19}H_{24}O_2N_4Br_2$	44·3	44·0	5·0	5·6			212–4
C4.10.4	$C_{22}H_{32}O_2N_4Br_2$					10·3	9·6	210–2

(Michel, 1949). In order to measure the total possible extent of reactivation 2 ml. of inhibited enzyme suspension was incubated with 2 ml. 0.02M hydroxyiminoacetone in barbitone buffer for 6 hr. at 25°, when reactivation was effectively complete (Davies and Green, 1956).

Animal Experiments.—All the experiments were carried out on female mice weighing 15 to 25 g. Saline solutions of ethyl pyrophosphate or sarin (isopropyl methylphosphonofluoridate) were injected in a volume of 10 ml./kg. subcutaneously into the loose skin at the back of the neck. The oxime (for doses see tables) and atropine sulphate, 17.4 mg./kg., were given intramuscularly in a volume of 3 ml./kg. into the outer aspect of the hind leg, either 10 min. before or 1 min. after injection of the organophosphate.

RESULTS

Inhibition and Reactivation of Cholinesterase

Table II shows the inhibitory effect of diquaternary oximes on erythrocyte cholinesterase. At 2×10^{-5}M only those compounds in which the two quaternary nitrogen atoms are separated by six or more carbon atoms show any significant inhibition. This increase of inhibitory power with increase in chain length is similar to that found with other diquaternary alkanes (Paton and Zaimis, 1949). Reactivation was carried out with an oxime concentration of 2×10^{-5}M. For assay, the solution was diluted tenfold, thereby reducing the oxime concentration to 2×10^{-6}M. At this concentration, only C4.10.4 gave any measurable inhibition and that only 40%, so any apparent failure to reactivate could not have been due to inhibition by the oxime during assay.

Interpretation of the reactivation experiments is made difficult by the apparent complexity of the kinetics (Fig. 1). Many of the compounds caused fairly rapid reactivation during the first few minutes of contact with the inhibited enzyme, but then reactivation apparently ceased or continued only very slowly (Curve **B**). The level of this apparent plateau in the rate curve for reactivation varied with the concentration of the oxime. Even with those compounds which gave complete reactivation and showed pseudo-unimolecular kinetics (Curve **A**) the apparent second-order rate constant varied with concentration as was found earlier with 2-hydroxyiminomethyl-*N*-methylpyridinium iodide (Green and Smith, 1958). It was thus not possible to use normal kinetic constants as a measure of comparative reactivating power. It was necessary instead to choose some arbitrary parameter to give some indication of the actual relative reactivating potencies. A fixed

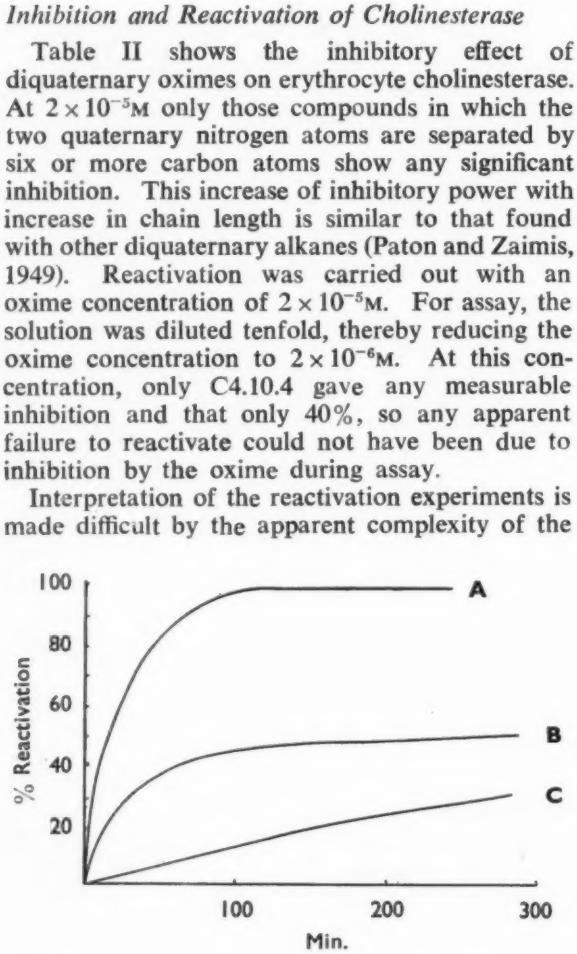

FIG. 1.—Reactivation of cholinesterase inhibited with ethyl pyrophosphate by oximes in concentrations of 2×10^{-5} M, *p*H 7.4 and 25°. **A**: C2.6.2. **B**: C4.3.IQ. **C**: C3.6.3.

TABLE II

INHIBITION OF CHOLINESTERASE, AND REACTIVATION OF DIETHYLPHOSPHORYL-CHOLINESTERASE, BY DIQUATERNARY OXIMES

The concentration used 2×10^{-5} M, *p*H 7·4 and 25°. Compounds marked n.t., not tested.

Compound		Aldoxime Positions or End Group	Chain Length	% Inhibition	Time Required for 25% Reactivation (min.)
2-Hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate				0	60
4-Hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate				0	>300
Group 1 Polymethylene 4,4′-dialdoximes	C4.2.4	4,4′	2	0	5
	C4.3.4	4,4′	3	0	5
	C4.4.4	4,4′	4	0	7
	C4.6.4	4,4′	6	32	25
	C4.10.4	4,4′	10	88	>300
Group 2 Trimethylene dialdoximes	C2.3.2	2,2′	3	0	120
	C3.3.3	3,3′	3	n.t.	220
	C4.3.4	4,4′	3	0	5
Group 3 Hexamethylene dialdoximes	C2.6.2	2,2′	6	32	4
	C3.6.3	3,3′	6	48	180
	C4.6.4	4,4′	6	32	25
Group 4 Monoaldoximes with *iso*quinolylpropyl chain	C2.3.IQ	2	3	n.t.	30
	C3.3.IQ	3	3	,,	>300
	C4.3.IQ	4	3	0	15
Group 5 Trimethylene 4-aldoximes with different end groups	C4.3.4	Pyridinium-4-aldoxime (4,4′)	3	0	5
	C4.3.P	Pyridinium	3	n.t.	13
	C4.3.IQ	Isoquinolinium	3	0	15
	C4.3.NEt_3	Triethylammonium	3	0	130
	C4.3.ST	Thiouronium	3	0	>300

reactivator concentration of 2×10^{-5}M has been used, and the parameter selected was the time required for 25% of the total inhibited enzyme to be reactivated. With all the compounds that gave a plateau effect, as in Curve **B**, the level of the plateau at this concentration was above 25%, and no reactivators which give only an initially rapid reactivation have been excluded by choosing this parameter. It is probable that 25% recovery of the cholinesterase activity *in vivo* would be more than sufficient to maintain life (Kewitz and Nachmansohn, 1957; Rutland, 1958). The times taken for 25% reactivation are given in Table II.

The effect of the methylene chain length upon reactivating power varied with the position of the hydroxyiminomethyl group in the pyridine ring. In the 4,4′-dioxime series (C4.*n*.4) reactivating power fell as the chain length increased; in the 3,3′-dioxime series (C3.*n*.3) changing the chain length from 3 to 6 carbon atoms had no significant effect, whereas in the 2,2′-dioxime series (C2.*n*.2) increasing the chain length to the same extent very greatly increased the reactivating power. The compounds with the hydroxyiminomethyl group in the 3-position in the pyridine ring were in general very much inferior to those with the hydroxyiminomethyl group in the 2- or 4-position. This might be expected from earlier work with the simple hydroxyiminomethyl-*N*-methylpyridinium iodides (Green and Smith, 1958).

The Toxicity of Diquaternary Oximes.—Table III shows the approximate maximum non-lethal dose of most of the oximes given subcutaneously. The toxicity generally increased with increasing methylene chain length, although even with a fixed chain length there was some variation which depended on the nature of R+. No obvious signs of poisoning were noticed at 5 mg./kg. with any of these compounds, while some of the more promising compounds were not toxic at 50 mg./kg. As with 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate (Davies and Willey, 1958), lethal doses caused muscular tremors, convulsions, cyanosis and death within 15 to 30 min.

TABLE III

TOXICITY TO MICE OF SOME DIQUATERNARY OXIMES

The dose recorded is the maximum at which no deaths occurred. The oximes were given subcutaneously to groups of 5 mice.

Dose (mg./kg.)			
5	10	20	50
C4.10.4	C4.6.4		C4.2.4
			C4.3.4
C2.6.2			C2.3.2
	C3.6.3		C3.3.3
		C4.3.IQ	C3.3.IQ
		$C4.3.NEt_3$	C4.3.P
			C4.3.MP
			C4.3.ST

TABLE IV

EFFECT OF OXIMES ON THE TOXICITY OF ETHYL PYROPHOSPHATE TO MICE

5 mg./kg. of the oxime plus 17·4 mg./kg. atropine sulphate were given intramuscularly 10 min. before subcutaneous ethyl pyrophosphate. Number of deaths/group of 6 mice is recorded. Ethyl pyrophosphate alone caused no deaths at 0·25 mg./kg. and 6 deaths at 0·5 mg./kg.

		Dose of Ethyl Pyrophosphate (mg./kg.)						
		1	2	4	8	16	32	64
Oxime								
2-Hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate		0	2	5				
4-Hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate		5	6					
Group 1	C4.2.4 ..				0	3	6	
	C4.3.4 ..					1	1	4
	C4.4.4 ..				1	4	5	
	C4.6.4 ..		0	3	6	5	5	6
	C4.10.4 ..	2	4	5	6			
,, 2	C2.3.2 ..		3	5	6	6		
	C3.3.3 ..	1	3	5	4	6		
	C4.3.4 ..					1	1	4
,, 3	C2.6.2 ..				0	0	2	2
	C3.6.3 ..			1	2	6		
	C4.6.4 ..		0	3	6	5	5	6
,, 4	C2.3.IQ ..		1	5	4	6		
	C3.3.IQ ..		5	6	5	6	6	
	C4.3.IQ ..				1	3	4	4
,, 5	C4.3.4 ..					1	1	4
	C4.3.P ..				1	3	4	6
	C4.3.MP				0	2	4	6
	C4.3.IQ ..				1	3	4	4
	$C4.3.NEt_3$		1	5	6	6		
	C4.3.ST ..		6	6				

Effect of the Oximes upon the Toxicity of Ethyl Pyrophosphate to Mice.—Table IV shows the effect of 5 mg./kg. of the oximes, given with 17.4 mg./kg. of atropine sulphate 10 min. before various doses of ethyl pyrophosphate. With few exceptions the compounds were at least as effective as 2 - hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate and C4.3.4 and C2.6.2 were outstandingly good. The effect of increasing the methylene chain length varied with the position of the hydroxyiminomethyl group. In the series C4.*n*.4 (Group 1) the best had a 3-carbon chain, whereas with both series C2.*n*.2 and C3.*n*.3 (Groups 2 and 3) six methylene groups were better than three. Of the unsymmetrical oximes (Group 5) those in which the second charged group R+ is a quaternary nitrogen heterocyclic group were much better than those in which it is a thiouronium or triethylammonium group.

TABLE V

EFFECT OF OXIMES ON TOXICITY OF ETHYL PYROPHOSPHATE TO MICE

10 mg./kg. of the oxime plus 17·4 mg./kg. of atropine sulphate were given intramuscularly 1 min. after ethyl pyrophosphate, subcutaneously. Number of deaths in groups of 10 animals is recorded. Ethyl pyrophosphate alone caused no deaths at 0·32 mg./kg., 10 deaths at 0·50 mg./kg. At 10 mg./kg. the animals collapsed before oxime and atropine could be given.

Oxime	Dose of Ethyl Pyrophosphate (mg./kg.)				
	0·8	1·6	3·2	6·3	8·0
2-Hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate	0	7	10		
C4.3.4		0		3	4
C4.3.P			3	3	7

The oximes have been given 1 min. after ethyl pyrophosphate in only a few experiments (Table V). Very good results were again obtained, particularly with C4.3.4. With large doses of ethyl pyrophosphate in excess of twenty times the LD50, the animals collapsed or died before the oxime could be administered. Of those animals which had not collapsed within the first minute, a considerable proportion recovered after treatment.

Effect of Oximes on the Toxicity of Sarin to Mice.—A few of the more promising compounds in the treatment of ethyl pyrophosphate poisoning were also given to mice 10 min. before sarin. Although they were better than 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate (Table VI) the improvement was not as marked as after ethyl pyrophosphate.

A more extended series of tests was carried out giving the oximes one minute after poisoning by sarin (Table VII). Most of them were slightly better than 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate, but none was outstandingly superior.

TABLE VI

EFFECT OF OXIMES ON THE TOXICITY OF SARIN TO MICE

10 mg./kg. of oxime plus 17·4 mg./kg. atropine sulphate were given intramuscularly 10 min. before sarin, subcutaneously. The number of deaths in groups of 6 mice is recorded. Sarin alone caused 1 death at 0·2 mg./kg. and 6 deaths at 0·4 mg./kg. C2.6.2 was tested at 5 mg./kg., since 10 mg./kg. killed all mice in 10 to 12 min.

Oxime	Dose of Sarin (mg./kg.)				
	0·2	0·4	0·8	1·6	3·2
2-Hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate	0	2	5		
C4.3.4		1	3	5	5
C4.3.P		0	5	5	6
C4.3.NEt$_3$	0	1	5	6	
C2.6.2	1	3	2	3	

TABLE VII

EFFECT OF OXIMES ON TOXICITY OF SARIN TO MICE

10 mg./kg. of oxime plus 17·4 mg./kg. of atropine sulphate were given intramuscularly 1 min. after sarin, subcutaneously. The number of deaths in groups of 10 mice is recorded. Sarin alone caused 1 death at 0·16 mg./kg. and 10 deaths at 0·32 mg./kg.

Oxime		Dose of Sarin (mg./kg.)			
		0·32	0·40	0·50	0·63
2-Hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate		4	8	9	
Group 1	C4.2.4	2	5	7	5
	C4.3.4	0	2	4	8
	C4.6.4	1	1	1	7
	C4.10.4	4	7	9	7
,, 2	C2.3.2	0	4	7	9
	C3.3.3	0	2	4	8
	C4.3.4	0	2	4	8
,, 3	C2.6.2	4	7	9	7
	C3.6.3	2	2	7	7
	C4.6.4	1	1	1	7
,, 5	C4.3.4	0	2	4	8
	C4.3.P	0	3	5	8
	C4.3.MP	3	3	6	8
	C4.3.IQ	1	1	6	8
	C4.3.NEt$_3$	2	5	6	9
	C4.3.ST	8	10		

DISCUSSION

Many oximes derived from $\alpha\omega$-diquaternary alkanes have been shown to be better than 2 - hydroxyiminomethyl - *N* - methylpyridinium methanesulphonate, when given at the same dose and with atropine, in saving the lives of mice poisoned with ethyl pyrophosphate. The best of these, C4.3.4 {*NN'* - trimethylenebis(4 - hydroxyiminomethylpyridinium bromide)}, at only 5 mg./kg. raised the LD50 of ethyl pyrophosphate about one hundred fold, whereas the former substance at this dose raised the LD50 only fivefold. In mice, these new oximes are much less effective against sarin poisoning than against ethyl pyrophosphate poisoning, but by analogy with 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate this may not be the case in other species: the latter is far more effective against sarin in rabbits and guinea-pigs than it is in mice (Davies *et al.*, 1959).

Using the time required for recovery of 25% of the enzyme activity (t_{25}) as a basis of comparison many of these newer oximes are better reactivators than 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate. Among the best are the trimethylene 4,4′-dioxime (C4.3.4) and the hexamethylene 2,2′-dioxime (C2.6.2), which are also the most effective in treatment. This relationship between life-saving and reactivating properties is fairly general to all the compounds investigated. Fig. 2 shows the relationship

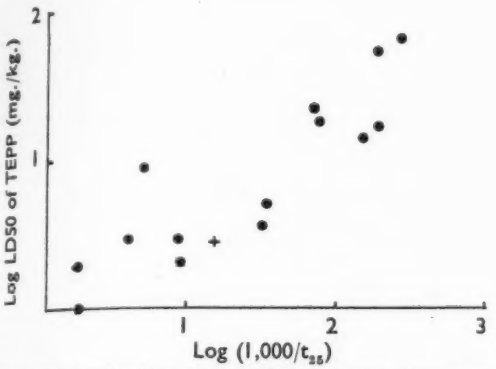

FIG. 2.—Relationship between life-saving and reactivating powers of diquaternary oximes. LD50 of ethyl pyrophosphate (TEPP) calculated from Table IV; t_{25} taken from Table II. + Indicates results for 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate.

between log (LD50) of ethyl pyrophosphate when given 10 min. after the oxime and atropine, and log (1,000/t_{25}).

There appears to be no simple relationship between these biological properties and chemical structure. It is clear, however, that the belief that the 2 - hydroxyiminomethyl-*N*-methylpyridinium salts are unique, owing their high reactivating power to their ability to fit precisely on to the surface of inhibited cholinesterase (Nachmansohn, 1958) is no longer tenable, since in the series examined here considerable variation in structure may be made without affecting their superiority as reactivators.

The good relationship between the therapeutic activity of these oximes and their reactivating powers strongly suggests that the reactivation of inhibited cholinesterase is the primary therapeutic action of these compounds. There are, however, two other properties which could contribute to this therapeutic action. Firstly, these compounds are inhibitors of cholinesterase, and if this inhibition is reversible, as would be expected from their structure (Austin and Berry, 1953), they may have a protective action against irreversible inhibitors similar to that displayed by eserine (Koelle, 1946). However, the lack of any correlation between anticholinesterase and therapeutic action in the present series (see Tables II and IV) makes this unlikely. Secondly, because these oximes have a diquaternary structure, they may block acetylcholine as does hexamethonium, which has been used successfully as an adjuvant to atropine (Parkes and Sacra, 1954). The present compounds have not yet been tested for such actions, but, as the therapeutic activity and reactivating power have been shown to be closely related, it is unlikely that such actions would be of more than secondary importance in this group of compounds as a whole.

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PROTECTION AGAINST LETHAL ORGANOPHOSPHATE POISONING BY QUATERNARY PYRIDINE ALDOXIMES

BY

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The effect of 18 pyridinium aldoximes on diethylphosphoryl-acetocholinesterase *in vitro* and the protection against lethal poisoning by ethyl pyrophosphate (TEPP) in mice pretreated with 0.095 m.mole/kg. of these oximes was investigated. Monoximes and dioximes of polymethylene-bispyridinium compounds were studied in greater detail since they were up to 22 times more potent than pyridine-2-aldoxime methiodide (2-hydroxyiminomethyl-*N*-methylpyridinium iodide) in reactivating diethylphosphoryl-acetocholinesterase *in vitro* and protected mice against lethal poisoning by up to 15 LD100 of ethyl pyrophosphate. These oximes were also up to 52 times more potent than pyridine-2-aldoxime methiodide in reactivating di-isopropylphosphoryl-acetocholinesterase *in vitro* and were effective in preventing lethal poisoning by dyflos (di-isopropyl phosphorofluoridate). The antidotal action against diethyl phosphostigmine (Ro 3-0340) was even greater than that against ethyl pyrophosphate. Some of the most effective oximes had antidotal actions in poisoning by ethyl pyrophosphate, diethyl phosphostigmine and dyflos when given in 0.0095 m.mole/kg. and this effect was enhanced by 1 mg./kg. atropine sulphate. *In vivo* reactivation of diethylphosphoryl-acetocholinesterases by 0.0095 or 0.095 m.mole/kg. of oximes of polymethylenebispyridinium compounds was demonstrated in blood but not in brain. Atropine-like and neuromuscular blocking activities were studied on isolated organs and protection against lethal doses of neostigmine and related anticholinesterases were also investigated. Some of the oximes of polymethylenebispyridinium compounds have, relative to pyridine-2-aldoxime methiodide, a higher therapeutic ratio in mice and considerably greater water-solubility. The possible advantages to be gained from their use in preference to pyridine-2-aldoxime methiodide are discussed.

The interaction between anticholinesterases of the organophosphate type and acetocholinesterase (also often referred to as true cholinesterase or acetylcholinesterase) yields phosphorylated aceto-cholinesterase which is enzymatically inactive and in many instances has a half-life ranging from several days to weeks. While cholinesterase is in a phosphorylated form acetylcholine accumulates and thus the administration of organophosphates is followed by muscarinic and nicotinic symptoms of acetylcholine poisoning.

Causative treatment of poisoning by certain organophosphates has recently become available as a result of the finding that some oximes, especially pyridine-2-aldoxime methiodide (2-hydroxyiminomethyl-*N*-methylpyridinium iodide ; P2AM), restore the activity of phosphorylated acetocholinesterase by dephosphorylation (Childs, Davies, Green, and Rutland, 1955 ; Wilson and Ginsburg, 1955). The successful treatment of parathion poisoning of man with pyridine-2-aldoxime methiodide has been reported by Namba and Hiraki (1958) and Namba (1958). From biochemical findings (Hobbiger, 1957a, b) it is clear, however, that the effectiveness of pyridine-2-aldoxime methiodide depends on the type of phosphoryl group attached to the enzyme. For equal degrees of enzyme reactivation the quantity of pyridine-2-aldoxime methiodide required when a di-isopropylphosphoryl group is involved, as for instance in poisoning with dyflos (di-isopropyl phosphorofluoridate), is 20 to 30 times higher than that necessary for the treatment of poisoning with parathion, ethyl pyrophosphate (TEPP) or paraoxon where a diethylphosphoryl group is involved. The dose of pyridine-2-aldoxime methiodide used and recommended by Namba and Hiraki (1958) for the treatment of

parathion poisoning is 1 g. or more, given intravenously. Because pyridine-2-aldoxime methiodide is only soluble in water to an extent of 4 to 5% at room temperature this means that at least 20 ml. of a hypertonic solution is required for reactivation of diethylphosphoryl-acetocholinesterase, and this treatment becomes impracticable where a di-isopropylphosphoryl group or another similarly strongly bound group is involved. It has also to be remembered that parathion itself does not phosphorylate acetocholinesterase but is converted into an active phosphorylating agent *in vivo* (Diggle and Gage, 1951), and thus produces symptoms more slowly than other organophosphates, such as ethyl pyrophosphate, paraoxon, or dyflos, which themselves are phosphorylating agents.

Since organophosphorus insecticides are used widely and cases of poisoning have frequently been reported (Pribilla, 1955; Holmes and Gaon, 1956; Conley, 1957; Maresch, 1957; Erdmann and Lendle, 1958; Namba and Hiraki, 1958; Quinby and Lemmon, 1958), reactivators of phosphorylated acetocholinesterase which have a low toxicity and which are more water-soluble and more potent than pyridine-2-aldoxime methiodide are urgently needed.

Methods and Materials

Potency of Oximes as Reactivators.—The potency of oximes as reactivators was determined by measuring their effect on diethylphosphoryl- and di-isopropylphosphoryl-acetocholinesterase at 37° in a medium of 0.025 M-$NaHCO_3$ (*p*H 7.4). Human red cells were used as source of acetocholinesterase. The two types of phosphorylated acetocholinesterase were obtained as described previously (Hobbiger, 1957b). Reactivation was studied in the Warburg apparatus, allowing 30 min. incubation of phosphorylated acetocholinesterase with the oxime in the centre compartment before addition of the substrate (acetylcholine chloride; final concentration 0.01 M) from the sidearm. The degree of reactivation was calculated from the CO_2 output between 5 and 35 min. after tipping in the substrate.

For toxicity tests and *in vivo* protection experiments, male white mice of an inbred strain and weighing 18 to 20 g. were used. For simplicity, the weight of each mouse was assumed to be 20 g. All injections were given in a volume of 0.2 ml. and normal saline was substituted when necessary to keep the volume of injected fluid and the number of injections constant between groups. All substances were dissolved in normal saline.

Determination of the LD50.—Oximes were given intraperitoneally using 6 mice for each dose and solutions which represented a geometrical progression of oxime concentrations with a difference of 1.5 between any given dose and the next higher dose. All dose/mortality curves were steep, and, in a certain range, mortality increased by at least 66% when the dose of the oxime was doubled. The point of intersection of the dose/mortality curve with the line representing death of half the mice was taken as the LD50.

In vivo *Protection Against Organophosphate Poisoning.* — The organophosphates and other anticholinesterases were injected subcutaneously in the middle of the back. The expression "complete protection" is used when all mice in a group survived for 24 hr., whereas the expression "partial protection" means that some mice in a group died within 24 hr.

Activity of Acetocholinesterase in Blood and Brain. —This was measured manometrically with 0.03 M-methacholine chloride as substrate (see Hobbiger, 1957a).

Experiments on Isolated Organs.—Antiacetylcholine and antihistamine actions were determined on isolated segments (3 to 4 cm.) of guinea-pig ileum suspended in oxygenated Tyrode solution at 37°. Oximes were added to the organ bath 30 sec. before acetylcholine or histamine and the concentration of the oximes required to reduce the sensitivity of the ileum by 50% was calculated from dose/antagonism curves.

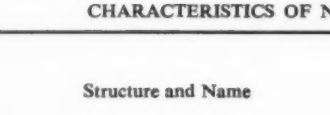
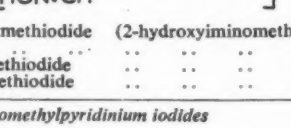
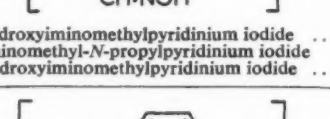
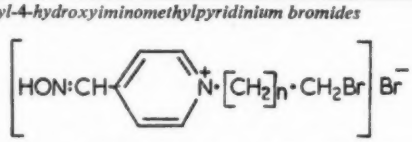
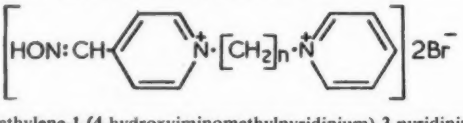
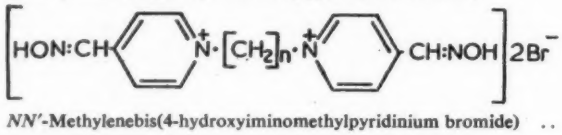
The isolated rat phrenic-nerve diaphragm was set up in the conventional manner (37°; oxygenated Tyrode solution) and the effect of the oximes on the response of the muscle to indirect stimulation by supramaximal rectangular pulses (duration, 0.25 msec.; frequency 1/10 sec.) was recorded. The effects obtained 10 min. after addition of an oxime to the organ bath were used in order to compare activities.

Organophosphates and Other Anticholinesterases.—Those used were (1) ethyl pyrophosphate (TEPP); (2) diethyl phosphostigmine {3-(diethoxyphosphinyloxy)-*NNN*-trimethylanilinium methosulphate, Ro 3-0340}; (3) dyflos (di-isopropyl phosphorofluoridate); (4) paraoxon (diethyl *p*-nitrophenyl phosphate, E 600); (5) neostigmine methylsulphate; (6) a bisneostigmine [*NN'* - decamethylenebis{3-(*N*-methylcarbamoyloxy)-*NNN*-trimethylanilinium bromide}, BC-48]; and (7) a bispyridostigmine [*NN'* - hexamethylenebis{3 - (*N* - methylcarbamoyloxy)-*N*-methylpyridinium bromide}, BC-51].

Oximes.—The oximes used were all pyridinium aldoximes synthesized in this laboratory. They are listed in Table I together with analytical information and solubilities in water and chloroform. The latter were determined spectroscopically in a Hilger-Uvispek using matched quartz cells. Absorption curves were determined in 0.01 N-NaOH.

Methods of Preparation: N - Alkyl - 2 - and - 4 - hydroxyiminomethylpyridinium Iodides.—The appropriate pyridine aldoxime (0.04 M) was dissolved in ethanol and heated under reflux for 24 hr. with an excess (0.08 M) of alkyl iodide. The ethanol and

TABLE I
CHARACTERISTICS OF NEW OXIMES SYNTHESIZED

Compound No.	Structure and Name	Required (%) C	Required (%) H	Found (%) C	Found (%) H	Molar Solubility in Water $\times 10^{-1}$	Molar Solubility in Chloroform $\times 10^{-5}$
	Pyridine aldoxime methiodides 						
—	Pyridine-2-aldoxime methiodide (2-hydroxyiminomethyl-*N*-methylpyridinium iodide)					1·8	2·8
2	Pyridine-3-aldoxime methiodide			(Known compounds)		>10	4·6
3	Pyridine-4-aldoxime methiodide					>10	5·4
	N-*Alkyl*-2-*hydroxyiminomethylpyridinium iodides* 						
4	$n=1$. *N*-Ethyl-2-hydroxyiminomethylpyridinium iodide					3·5	11·0
5	$n=2$. 2-Hydroxyiminomethyl-*N*-propylpyridinium iodide	37	4·5	37·3	4·7	0·7	12·8
6	$n=3$. *N*-Butyl-2-hydroxyiminomethylpyridinium iodide	39·2	4·9	39·3	5·2	0·3	24·0
							
7	Pyridine-4-aldoxime ethiodide (*N*-ethyl-4-hydroxyiminomethylpyridinium iodide)	34·5	3·9	34·4	3·9	>10	30
	N-*ω-Bromoalkyl*-4-*hydroxyiminomethylpyridinium bromides* 						
8	$n=2$. *N*-(3-Bromopropyl)-4-hydroxyiminomethylpyridinium bromide	33·3	3·7	33·3	3·8	0·7	2·2
9	$n=3$. *N*-(4-Bromobutyl)-4-hydroxyiminomethylpyridinium bromide	35·5	4·1	35·6	4·3	>10	11·0
10	$n=4$. *N*-(5-Bromopentyl)-4-hydroxyiminomethylpyridinium bromide	37·5	4·5	37·3	4·5	0·1	5·1
	Monoximes of NN′-*polymethylenebis*(*pyridinium bromides*) 						
11	$n=3$. Trimethylene-1-(4-hydroxyiminomethylpyridinium)-3-pyridinium dibromide	41·7	4·2	41·3	4·4	>10	0·53
12	$n=4$. Tetramethylene-1-(4-hydroxyiminomethylpyridinium)-4-pyridinium dibromide	43·2	4·5	43·5	4·7	>10	0·09
13	$n=5$. Pentamethylene-1-(4-hydroxyiminomethylpyridinium)-5-pyridinium dibromide	44·5	4·9	44·2	4·8	>10	0·32
	NN′-*Polymethylenebis*(4-*hydroxyiminomethylpyridinium bromides*) 						
14	$n=1$. *NN′*-Methylenebis(4-hydroxyiminomethylpyridinium bromide)	37·3	3·4	36·9	3·5	Decomposes in 0·01 N-NaOH	
15	$n=2$. *NN′*-Ethylenebis(4-hydroxyiminomethylpyridinium bromide)	38·9	3·7	38·8	3·9	0·3	0·06
16	$n=3$. *NN′*-Trimethylenebis(4-hydroxyiminomethylpyridinium bromide)	40·4	4·0	39·8	4·2	>10	0·08
17	$n=4$. *NN′*-Tetramethylenebis(4-hydroxyiminomethylpyridinium bromide)	41·7	4·4	41·8	4·6	2·8	0·54
18	$n=5$. *NN′*-Pentamethylenebis(4-hydroxyiminomethylpyridinium bromide)	43·0	4·6	42·8	4·8	>10	3·2

unreacted alkyl iodide were removed by distillation under reduced pressure and the products crystallized from ethanol or ethanol/ether.

N-ω-Bromoalkyl-4-hydroxyiminomethylpyridinium Bromides.—Pyridine-4-aldoxime (0.05 M) was dissolved in ethanol (50 ml.) and heated under reflux for 18 hr. with excess polymethylene dibromide (0.15 M). The mixture was cooled and filtered to remove any dioxime formed, then the ethanol and unreacted polymethylene dibromide were removed by distillation under reduced pressure. The residual crystals were triturated with ether and recrystallized from ethanol.

Monoximes of NN'-Polymethylenebis(pyridinium Bromides). — N-ω-Bromoalkyl-4-hydroxyiminomethylpyridinium bromide (0.02 M) was dissolved in ethanol (50 ml.) and heated under reflux for 18 hr. with excess pyridine. The ethanol and unreacted pyridine were removed by distillation under reduced pressure. The residual oil was triturated with ether and crystallized from ethanol.

NN' - Polymethylenebis(4-hydroxyiminomethylpyridinium Bromides).—Pyridine-4-aldoxime (0.1 M) was dissolved in ethanol and heated under reflux for 18 hr. with polymethylene dibromide (0.06 M). The product separated on cooling and was recrystallized from methanol. Compound 14 was an exception as it was the only product obtained from the attempted preparation of *N*-bromomethyl-4-hydroxyiminomethylpyridinium bromide.

A full account of biochemical studies on compounds 2 to 18 is in preparation.

Throughout the text, the oximes will be referred to by the compound numbers in Table I. Doses and concentrations are expressed in m.mole/kg. and molarities respectively. In earlier work (Hobbiger, 1957a) a dose of 25 mg./kg. pyridine-2-aldoxime methiodide (0.095 m.mole/kg.) was used and, to make the earlier results directly comparable with those of the present investigation, the unusual dose of 0.095 m.mole/kg. was retained for *in vivo* studies.

Two short preliminary accounts of some of the work presented here have been published (Hobbiger, O'Sullivan and Sadler, 1958; Hobbiger and Sadler, 1958). In the first of these, *NN'*-methylenebis(4-hydroxyiminomethylpyridinium bromide) (compound 14, Table I) was erroneously listed as *N*-bromomethyl-4-hydroxyiminomethylpyridinium bromide.

RESULTS

Reactivation of Phosphorylated Acetocholinesterase in vitro.—Table II shows the potency of the 18 derivatives of pyridinium aldoximes as reactivators of diethylphosphoryl-acetocholinesterase at *p*H 7.4. As can be seen, the hydroxyiminomethyl group is required in the 2-position for highest activity in the case of the methiodides of the pyridine aldoximes, and an increase in size of the alkyl group (compare pyridine-2-aldoxime methiodide and compound 3 with compounds 4 to 6 and 7 respectively) is associated with some loss of activity. The introduction of a terminal bromine atom in the alkyl chain (compounds 8 to 10) greatly enhanced activity in comparison with the parent compound: the monoximes (compounds 11 to 13) of polymethylenebispyridinium compounds were 6.7 to 8 times, and the dioximes (compounds 14 to 18) 2.8 to 22 times, more active than pyridine-2-aldoxime methiodide.

The high potency of compounds 11 to 13 and 15 to 18 as reactivators was not limited to diethylphosphoryl-acetocholinesterase but is even slightly greater with di-isopropylphosphoryl-acetocholinesterase (Table II).

TABLE II

IN VITRO REACTIVATION OF PHOSPHORYLATED ACETOCHOLINESTERASE AND TOXICITY IN MICE

The potency as reactivator was determined from rates of reactivation of (1) diethylphosphoryl and (2) di-isopropylphosphoryl-acetocholinesterase using $4–12 \times 10^{-6}$ and $5–15 \times 10^{-5}$ M pyridine-2-aldoxime methiodide respectively as reference (= 100). See Methods section for details. Relative toxicities were calculated from the LD50 which in the case of pyridine-2-aldoxime methiodide was 1 m.mole/kg. Oximes were injected intraperitoneally. All comparisons are on a molar basis.

Compound	Potency as Reactivator		Relative Toxicity
	(1)	(2)	
2	0·03		
3	6		
4	54		
5	63		
6	87		
7	3		
8	190		
9	25		
10	94		
11	800	1,100	200
12	670	1,000	280
13	750	1,100	620
14	282		
15	1,700	2,200	160
16	2,200	5,200	350
17	1,800	3,800	530
18	1,600	3,600	1,500

Effects in vivo

Toxicity.—Since it was our aim to find compounds which were superior to pyridine-2-aldoxime methiodide as antidotes of organophosphate poisoning, the only toxicities investigated were those of oximes which were at least six times more potent than pyridine-2-aldoxime methiodide as reactivators of diethylphosphoryl-acetocholinesterase *in vitro*. Table II shows that, on a molar basis, compounds 11 to 13 and 15 to 18 were 1.6 to 15 times more toxic than pyridine-2-aldoxime methiodide. However, using as therapeutic index the ratio LD50/concentration required for reactivation, most of the new oximes were safer to use than equi-effective doses of pyridine-2-aldoxime methiodide. Particularly

favourable therapeutic indices (2.4 to 15 higher than that of pyridine-2-aldoxime methiodide) were seen with compounds 11, 12, and 15 to 17.

Symptoms produced by lethal doses of compounds 11 to 13 and 15 to 18 were identical with those produced by pyridine-2-aldoxime methiodide, namely dyspnoea followed by convulsions and respiratory arrest. The latter always preceded cessation of the heart beat. With the exception of compound 18, all oximes were well tolerated when given intraperitoneally in a dose of 0.095 m.mole/kg. Since such a dose of compound 18 was lethal its antidotal action was not investigated.

Protection against Lethal Poisoning by Ethyl Pyrophosphate.—Compounds 1 to 17 were investigated for a protective action against lethal poisoning by 0.8 mg./kg. of ethyl pyrophosphate. This particular dose was chosen because it produced with certainty a lethal effect in otherwise untreated mice whereas more than 75% of the mice injected with half that dose survived. Complete protection by 0.095 m.mole/kg. of oxime against lethal poisoning by 0.8 mg./kg. of ethyl pyrophosphate was obtained only with oximes of polymethylenebispyridinium compounds. The latter, with the exception of compound 14, also gave either complete or partial protection against lethal poisoning by 1.6 to 3.2 mg./kg. of ethyl pyrophosphate. Compounds 13, 16, and 17 were the best antidotes, and partial protection against the lethal effect of 12.8 mg./kg. of ethyl pyrophosphate was obtained in mice pretreated with compound 16. These results are summarized in Table III.

TABLE III

PROTECTION AGAINST LETHAL POISONING BY ETHYL PYROPHOSPHATE

Mice received 0·095 m.mole/kg. of oxime intraperitoneally 5 to 10 min. before ethyl pyrophosphate (given subcutaneously). Results are number of mice surviving for 24 hr./number of mice injected.

Oxime	Ethyl Pyrophosphate (mg./kg.)						
	0·4	0·8	1·6	3·2	6·4	12·8	25·6
None ..	11/12	0/12	0/6	0/6	0/6	0/6	
Pyridine-2-aldoxime methiodide		0/6	0/18	0/12			
Compound 2		0/6	0/6				
,, 3		0/6	0/6				
,, 4		0/6	0/6				
,, 5		0/6	0/6				
,, 6		1/6	0/6				
,, 7		0/6	0/6				
,, 8		4/6	0/6				
,, 9		2/6	0/6				
,, 10		1/6	0/6				
,, 11		6/6	3/6	3/6	1/7		
,, 12		6/6	5/6	3/6	1/7		
,, 13		6/6	5/6	6/6	5/9	2/9	
,, 14		6/6	1/6	0/6			
,, 15		6/6	2/6	1/6	0/7		
,, 16		6/6	6/6	6/6	9/11	7/15	0/6
,, 17		6/6	6/6	5/6	8/9	1/9	

Death induced by organophosphates in mice pretreated with an oxime was always associated with all the symptoms of acetylcholine poisoning. In groups with partial protection and in groups receiving the next lower dose of ethyl pyrophosphate, the survivors generally also showed symptoms of acetylcholine poisoning, particularly of the nicotinic type which lasted for several hours. Mice pretreated with compounds 13, 16, and 17 and receiving 0.8 or 1.6 mg./kg. of ethyl pyrophosphate had only very transient and mild symptoms.

The degree of protection against ethyl pyrophosphate given by 0.095 m.mole/kg. of oxime is determined by the interval between the injection of the latter and the administration of the organophosphate. When 0.095 m.mole/kg. of compounds 11 to 13 and 15 to 17 were injected 60 min. earlier none of the mice survived 1.6 mg./kg. of ethyl pyrophosphate, and the interval between injection of the organophosphate and death was the same as that recorded in a group of mice which received 1.6 mg./kg. of ethyl pyrophosphate only.

Two of the most effective antidotes, compounds 13 and 16, were tried at lower doses; 0.0095 m.mole/kg. of either oxime gave definite protection (Table IV). However, mice injected with 0.00095 m.mole/kg. of compounds 13 and 16 were not protected against 0.7 mg./kg. ethyl pyrophosphate; they died at the same time as the mice in the control group.

Protection against Lethal Poisoning by Diethyl Phosphostigmine.—Both diethyl phosphostigmine and ethyl pyrophosphate form diethylphosphoryl-acetocholinesterase, but only the latter is hydrolysed by mammalian esterases with phosphatase activity (Burgen and Hobbiger, 1951; Hobbiger, 1956). Lethal doses of diethyl phosphostigmine cause death after a longer interval than corresponding doses of ethyl pyrophosphate. Thus the oxime can act over a longer period, and it has been suggested that for this reason 0.095 m.mole/kg. of pyridine-2-aldoxime methiodide is far more effective in protecting against diethyl phosphostigmine than against ethyl pyrophosphate (Hobbiger, 1957a). In agreement with such an interpretation was the finding that oximes of polymethylenebispyridinium compounds were also more effective antidotes of diethyl phosphostigmine than of ethyl pyrophosphate. Compounds 13 and 16 were given in a dose of 0.0095 m.mole/kg., and

TABLE IV

PROTECTION AGAINST LETHAL POISONING BY ORGANOPHOSPHATES

Mice received 0·0095 m.mole/kg. of oxime intraperitoneally 5 to 10 min. before the organophosphate (given subcutaneously). Atropine sulphate (1 mg./kg.) was given intraperitoneally 15 min. before the oxime. Results are number of mice surviving for 24 hr./number of mice injected. Dose of organophosphate in mg./kg.

Oxime	Ethyl Pyrophosphate					Diethyl Phosphostigmine							Dyflos				
	0·35	0·7	1·4	2·8	5·6	5	10	20	40	80	160	320	3·8	5	7·5	10	20
(1) Without atropine:																	
None	6/6	1/6	0/6			5/6	0/6	0/6					6/6	0/6	0/6		
Pyridine-2-aldoxime methiodide		1/6	0/6				0/6	0/6						0/6			
Compound 11														3/6	0/6		
,, 13		6/6	0/6					6/6	6/6	5/6	1/6	0/6					
,, 16		5/6	0/6					6/6	6/6	6/6	3/6	0/6		5/6	3/6	0/6	
(2) With atropine:																	
None	6/6	2/6	0/6			6/6	1/6	0/6					6/6	0/6			
Pyridine-2-aldoxime methiodide		5/6	0/6				6/6	3/6	0/6					0/6			
Compound 11		6/6	5/6	0/6										4/6	3/6	0/6	
,, 13		6/6	4/6	0/6						6/6	4/6	0/6					
,, 16		6/6	6/6	3/6	0/6					6/6	6/6	1/6		6/6	6/6	5/6	1/6

Table IV summarizes the remarkable degree of protection against lethal poisoning by diethyl phosphostigmine obtained with the two oximes.

Protection against Lethal Poisoning by Dyflos.—Only pyridine-2-aldoxime methiodide and oximes of polymethylenebispyridinium compounds were used. All mice injected with 0.095 m.mole/kg. of compounds 11 to 13 and 15 to 17 survived a dose of dyflos which produced a 100% mortality in the control group (Table V). Compounds 11, 16, and 17 were the most effective antidotes; they gave partial protection against 3 to 6 LD100 of dyflos.

Cholinergic symptoms of oxime-treated mice surviving 10 to 20 mg./kg. of dyflos were always marked and long-lasting but delayed in onset by comparison with the symptoms in mice of a control group receiving only dyflos.

TABLE V

PROTECTION AGAINST LETHAL POISONING BY DYFLOS

Mice received 0·095 m.mole/kg. oxime intraperitoneally 5 to 10 min. before dyflos (given subcutaneously). Results are number of mice surviving for 24 hr./number of mice injected. The stock solution of dyflos used in these experiments had undergone approximately 40% hydrolysis and for this reason the toxicity of dyflos is lower than that observed in experiments presented in Table IV and in a previous paper (Hobbiger, 1957a). Repetition of the experiment using a limited number of mice and a fresh stock solution of dyflos established the validity of the observations presented in this Table.

Oxime	Dyflos (mg./kg.)					
	5	7·5	10	20	40	80
None	6/6	2/6	0/6	0/6	0/6	
Pyridine-2-aldoxime methiodide		6/6	2/6	0/6	0/6	
Compound 11			6/6	6/6	2/6	0/6
,, 12			6/6	4/6	0/6	
,, 13			6/6	5/6	0/6	
,, 15			6/6	3/6	0/6	
,, 16			6/6	6/6	3/6	0/6
,, 17			6/6	5/6	1/6	0/6

Two of the most effective oximes, compounds 11 and 16, were given in doses of 0.0095 m.mole/kg. Some protection against lethal poisoning by dyflos was also obtained with this lower dose (Table IV).

The Effect of Atropine on the Antidotal Action of Oximes.—Marked enhancement of the antidotal effect of pyridine-2-aldoxime methiodide by high doses of atropine sulphate (10 mg./kg. or more) has been repeatedly demonstrated (Kewitz, Wilson, and Nachmansohn, 1956; Askew, 1957; Hobbiger, 1957a). Since mice tolerate weight for weight more than 100 times the amount of atropine fatal to adult man (Gordon and Frye, 1955) experiments with low doses of atropine should be more important than experiments with large doses of atropine. For this reason mice were given only 1 mg./kg. of atropine sulphate 15 min. before the injection of 0.0095 m.mole/kg. of an oxime. Such a dose of atropine markedly reduced the intensity of muscarinic symptoms after injection of organophosphates but had no antidotal effect on its own. Mice pretreated with atropine and compounds 11, 13, or 16, however, survived larger doses of ethyl pyrophosphate, diethyl phosphostigmine or dyflos than mice pretreated with the oximes only (Table IV).

In vivo *Reactivation of Phosphorylated Acetocholinesterase.* — Speedy reactivation of diethylphosphoryl-acetocholinesterase in blood could be demonstrated in mice which were given 0.0095 or 0.095 m.mole/kg. of compounds 11 to 13 or 15 to 17, 30 min. after a sublethal dose of ethyl pyrophosphate (Table VI). Similarly, enzymatic activity was always high in the blood of mice which as the result of pretreatment with oximes had survived doses of ethyl pyrophosphate

greatly in excess of the LD100. For example, 24 hr. after the injection of ethyl pyrophosphate mice which received first 0.095 m.mole/kg. of compounds 13, 16, or 17, and 5 to 10 min. later 6.4 or 12.8 mg./kg. of ethyl pyrophosphate (survivors of experiments summarized in Table III), had an enzymatic activity in blood which varied between 70 and 81% of that of untreated control mice.

TABLE VI

IN VIVO REACTIVATION OF DIETHYLPHOSPHORYL-ACETOCHOLINESTERASE IN BLOOD

Mice were injected subcutaneously with 0·4 mg./kg. of ethyl pyrophosphate and 30 min. later the oxime was given intraperitoneally. Each result represents the enzyme activity of a pooled sample obtained from 3 mice 1 or 3 hr. after the injection of the oxime. Activities at 1 hr. after the injection are suspected of being slightly above their true value because of reactivation *in vitro* (Hobbiger, 1957a).

Oxime	Dose (m.mole/kg.)	Activity of Acetocholinesterase as % of Control	
		1 hr.	3 hr.
None	—	12	13
Pyridine-2-aldoxime methiodide	0·0095	29	—
	0·095	49	53
Compound 11	0·0095	53	56
	0·095	54	54
,, 12	0·095	56	60
,, 16	0·00095	32	—
	0·0095	54	52
	0·095	55	59
,, 17	0·095	47	56

On the other hand, no reactivation of phosphorylated acetocholinesterase in brain could be demonstrated with certainty in mice which received a sublethal dose of 0.4 mg./kg. of paraoxon or 1.25 to 2.5 mg./kg. of dyflos followed 30 min. later by 0.095 m.mole/kg. of compound 16.

Protection against Lethal Poisoning by Neostigmine and Related Anticholinesterases.— Neostigmine, the bisneostigmine (BC-48) and the bispyridostigmine (BC-51) are potent anticholinesterases which do not inhibit cholinesterase by phosphorylation. The biochemical and pharmacological actions of the last two have been described in detail by Kraupp, Stumpf, Herzfeld, and Pillat (1955) and Herzfeld, Kraupp, Pateisky, and Stumpf (1957). To test the specificity of the antidotal action of oximes of polymethylenebispyridinium compounds in organophosphate poisoning 0.095 m.mole/kg. of compound 16 were given to mice 5 to 10 min. before neostigmine, BC-48 or BC-51. Protection was most marked when neostigmine was used, but very little protection was obtained against BC-51 and none against BC-48. With neostigmine, 0.095 m.mole/kg. of compound 16 raised the LD100 approximately three-fold. These results are summarized in Table VII.

No protection against lethal neostigmine poisoning was found with 0.0095 m.mole/kg. of compound 16.

TABLE VII

EFFECT OF OXIMES ON LETHAL POISONING BY NEOSTIGMINE AND RELATED ANTICHOLINESTERASES

Mice were given the oxime intraperitoneally 5 to 10 min. before the anticholinesterase subcutaneously. Results are number of mice surviving for 24 hr./number of mice injected. The doses of neostigmine, BC-48 and BC-51 are in mg./kg.

Oxime	Dose (m.mole/kg.)	Neostigmine Methylsulphate				
		0·5	0·75	1	2	3
None	—	5/6	0/6	0/6		
Pyridine-2-aldoxime methiodide	0·095		0/6	0/6		
Compound 16	0·095		6/6	6/6	0/6	
,, 16	0·0095		0/6			
		Bisneostigmine (BC-48)				
		0·025	0·05	0·1		
None ..	—	6/6	1/6	0/6		
Compound 16	0·095		1/6	0/6		
		Bispyridostigmine (BC-51)				
		0·2	0·4	0·6		
None	—	6/6	3/6	0/6		
Compound 16	0·095		6/6	1/6	0/6	

Actions on Isolated Organs. — Since both atropine and tubocurarine give some protection against lethal organophosphate poisoning (Parkes and Sacra, 1954), the effect of compounds 11 to 13 and 15 to 17 on the sensitivity of the isolated ileum to acetylcholine and the response to indirect stimulation of the isolated rat phrenic-nerve diaphragm preparation were investigated.

Oximes reduced the sensitivity of the guinea-pig ileum to acetylcholine. The most effective compound *in vivo*, namely compound 16, was the most potent antagonist of acetylcholine on the ileum (Table VIII). Antagonism to histamine could also be demonstrated, but the concentrations required to obtain the same effect on equiactive doses of histamine and acetylcholine were at least 10 times greater for the former than the latter (Table VIII). In each case speedy recovery followed the removal of oximes from the organ bath.

In suitable concentrations the oximes reduced the twitch tension of the indirectly stimulated diaphragm (Table VIII). With the first dose of an oxime a just noticeable and transient increase of twitch tension often preceded paralysis. After

TABLE VIII
EFFECTS OF OXIMES ON ISOLATED ORGANS

Oxime	Guinea-pig Ileum Concentrations (mM) giving 50% Reduction in Sensitivity		Rat Phrenic-nerve Diaphragm Concentrations (mM) giving 70% Paralysis in 10 min.
	Acetylcholine	Histamine	
Pyridine-2-aldoxime methiodide ..	0·11		4·0
Compound 11 ..	0·06		1·7
" 12 ..	0·16		1·2
" 13 ..	0·054	>0·5	0·59
" 15 ..	0·32		2·00
" 16 ..	0·013	0·12	0·85
" 17 ..	0·068	>0·5	0·98
" 18 ..	0·035	>0·5	0·44

removal of the oxime from the organ bath full recovery of twitch tension was always rapid. Edrophonium (0.025 mM), which has a marked anti-curare action on the isolated rat phrenic-nerve diaphragm preparation (Hobbiger, 1952), increased twitch tension transiently by only 7% when added to the organ bath after compound 16 (within 6 min.) had reduced the twitch tension by 40%.

Effect on Alkylated "Receptors" and Energy-rich Phosphates.—Segments of guinea-pig ileum were bathed for 10 min. in 0.7 mM phenoxybenzamine. This resulted in a long-lasting reduction of their sensitivity to histamine as is typical for the effect of β-haloalkylamines (Nickerson, 1956). Addition of compound 16 in a final concentration of 2 mM to the organ bath for 10 min. failed to increase the sensitivity to histamine of the ileum treated with phenoxybenzamine.

Hydrolysis of adenosine triphosphate by pyridine-2-aldoxime methiodide, compound 3 and compound 16 was determined spectroscopically by incubating the oximes with adenosine triphosphate in barbitone buffer (*p*H 7.6) at 37° and developing with Fiske-Subbarow reagent. With a concentration of 2 mM of compounds 3 and 16 hydrolysis of adenosine triphosphate, as judged by the amount of inorganic phosphate present, amounted to 0 and 5% respectively after 15 min. incubation. Similarly pyridine-2-aldoxime methiodide and compound 16 in a concentration of 20 mM produced 1 and 6% hydrolysis respectively after 30 min. incubation.

DISCUSSION

A study of derivatives of pyridine aldoxime has led to oximes more potent than pyridine-2-aldoxime methiodide as reactivators of diethylphosphoryl and di-isopropylphosphoryl-acetocholinesterases. Particularly outstanding as reactivators are certain monoximes and dioximes of polymethylenebispyridinium compounds; for example, compounds 11 to 13 and 15 to 18 (Table II). Compounds 11, 12, 16, and 17 are more water-soluble, and in mice have a greater therapeutic ratio than pyridine-2-aldoxime methiodide; they are thus preferable to compounds 13, 18, and 15, which have the highest toxicity and lowest water-solubility respectively amongst the oximes of polymethylenebis-pyridinium compounds investigated. Should the toxicity in man of compounds 11, 12, 16, and 17, relative to pyridine-2-aldoxime methiodide, be similar to that found in mice (Table II) rates of reactivation of diethylphosphoryl-acetocholinesterase greatly in excess of those obtainable with pyridine-2-aldoxime methiodide could be accomplished by their administration to man. It might also be possible to reactivate freshly formed human di-isopropylphosphoryl-acetocholinesterase *in vivo*. Since compounds 11, 12, and 16 can be dissolved rapidly in a small volume of water they could be self-administered, in conjunction with atropine.

Oximes of polymethylenebispyridinium compounds, like pyridine-2-aldoxime methiodide, are relatively insoluble in oil (Table I); thus their effect will be exerted mainly outside the central nervous system and there particularly on cell surfaces. The first of these conclusions is supported by the finding that large doses of compound 16, like pyridine - 2 - aldoxime methiodide (Hobbiger, 1957a; Kewitz and Nachmansohn, 1957; Rutland, 1958), have no measurable effect on phosphorylated acetocholinesterase in the brain of mice although speedy reactivation takes place in blood (Table VI). Since these results were obtained by measuring the acetocholinesterase activity of the whole brain it is possible that compound 16 and related oximes nevertheless reactivate phosphorylated acetocholinesterase in a few limited areas of the brain which might play a vital part in survival. Preferential action on cell surfaces has been shown with pyridine-2-aldoxime methiodide (Fleisher, Corrigan, and Howard, 1958), and is characteristic of compounds which have a quaternary nitrogen atom (Schoffeniels, Wilson, and Nachmansohn, 1958). This limitation does not discredit reactivation of phosphorylated acetocholinesterase as the major or sole component of the mechanism by which the oximes act as antidotes, since the functional acetocholinesterase is located on cell surfaces (Koelle and Steiner, 1956; Koelle, 1957).

Whereas there is at present no evidence that the rate of reactivation *in vivo*, by a given oxime, of phosphorlyated acetocholinesterase at different sites depends upon the species studied, species differences in the antidotal effects of certain oximes have been clearly established (Askew, 1956). For this reason, it is advisable not to generalize on the degree of protection obtained with the oximes of polymethylenebispyridinium compounds described. In mice complete protection against the lethal effect of approximately two LD100 or higher doses of ethyl pyrophosphate by 0.095 m.mole/kg. of oxime was obtained only with those pyridine aldoximes which were more than twice as effective as pyridine-2-aldoxime methiodide in reactivating phosphorylated acetocholinesterase, namely the oximes of polymethylenebispyridinium compounds (compounds 11 to 17). Some of the latter in a dose of 0.095 m.mole/kg. protected mice against the lethal effect of 10 to 15 LD100 of ethyl pyrophosphate (Table III), and, as with pyridine-2-aldoxime methiodide (Hobbiger, 1957a), protection against lethal doses of diethyl phosphostigmine was even more marked (Table IV). Compounds 11, 16 and 17 were the most effective against lethal poisoning by dyflos and 0.095 m.mole/kg. protected some mice against 3 to 6 LD100 of dyflos (Table V). The antidotal effect of prophylactic doses of 0.0095 m.mole/kg. of oximes of polymethylenebispyridinium compounds was enhanced by 1 mg./kg. of atropine sulphate regardless of the type of organophosphate used (Table IV).

These results are encouraging and warrant an investigation, in species which are closely related to man, of the most effective oximes of polymethylenebispyridinium compounds (preferably in conjunction with atropine) as prophylactic agents against lethal doses of those organophosphates which form diethylphosphoryl- or di-isopropylphosphoryl-acetocholinesterases. If such experiments are carried out, attention must be paid to the rapidity with which the effect of the oximes wanes, a property which they share with pyridine-2-aldoxime methiodide (Kewitz, Wilson and Nachmansohn, 1956).

While this work was in progress Poziomek, Hackley, and Steinberg (1958) published the synthesis of compounds 4, 15, 16, 17, and 18 and reported that compound 16 is 5 times more potent *in vitro* than pyridine-2-aldoxime methiodide in reactivating isopropylmethylphosphoryl - aceto - cholinesterase. Bay, Krop, and Yates (1958) carried out experiments on a small number of mice, rabbits, cats and dogs giving compound 16 and pyridine-2-aldoxime methiodide, generally in conjunction with atropine, either before or shortly after sarin (isopropyl methylphosphonofluoridate) and found that the former was superior in all species except the dog.

Since atropine-like and neuromuscular blocking activities of pyridine-2-aldoxime methiodide have been demonstrated on isolated organs (Bethe, Erdmann, Lendle and Schmidt, 1957 ; Holmes and Robins, 1955), all oximes of polymethylenebispyridinium compounds were investigated for such properties. No relationship was found between the atropine-like activity of the oximes and their ability to paralyse indirectly stimulated striped muscle on the one hand and their effectiveness as antidotes on the other. An atropine-like action was most pronounced with compound 16 (Table VIII) and when 0.095 m.mole/kg. of this oxime is used the concentration reached *in vivo* might be sufficient to reduce the sensitivity of receptors for muscarinic actions of acetylcholine. It is unlikely that the other oximes have an atropine-like action *in vivo* at this concentration.

With regard to the significance of the effect on striped muscle for the antidotal action of some of the oximes, no valid conclusion can be drawn from the experiments on the isolated diaphragm since compounds containing a quaternary nitrogen atom are much less effective on this preparation than they are *in vivo* (Paton and Zaimis, 1952). However, there is a reasonably good relationship between the toxicity of oximes and their paralysing activity on indirectly stimulated striped muscle (compare Tables II and VIII).

Protection by 0.095 m.mole/kg. of compound 16 against lethal poisoning by neostigmine (Table VII) was much less than against lethal doses of ethyl pyrophosphate or diethyl phosphostigmine. The way in which the former was achieved is unknown, but it is possible that the atropine-like action of compound 16 accounts for it or is a contributory factor. No protection or only very little was obtained with 0.095 m.mole/kg. of compound 16 against lethal poisoning by the bisneostigmine (BC-48) and the bispyridostigmine (BC-51) respectively. The latter, like organophosphates, inhibit cholinesterase for much longer periods than does neostigmine (Kraupp, Stumpf, Herzfeld and Pillat, 1955 ; Herzfeld, Kraupp, Pateisky, and Stumpf, 1957), and thus it is questionable whether the mechanism responsible for protection against lethal neostigmine poisoning contributes significantly to protection against lethal organophosphate poisoning. In this connexion the findings of Grob and Johns (1958a and b) must

be mentioned. They observed that 1 to 2 g. of pyridine-2-aldoxime methiodide ameliorate generalized muscular weakness in man produced by neostigmine and the bispyridostigmine (BC-51) as well as by organophosphates. This indicates that in man improvements obtained by the treatment of non-lethal organophosphate poisoning with pyridine-2-aldoxime methiodide are not always entirely due to dephosphorylation. Our experiments with pyridine-2-aldoxime methiodide (Table VII) show that 0.095 m.mole/kg. of this oxime do not protect mice against lethal neostigmine poisoning.

In view of the high reactivity of oximes of polymethylenebispyridinium compounds, additional experiments were carried out to investigate if they could also restore the sensitivity of "receptors" which had been inactivated in an "irreversible" manner by alkylation (Nickerson, 1949) or if they interacted with energy-rich phosphates. Compound 16 was used for this purpose and found to be devoid of such properties.

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ABSORPTION, METABOLISM AND ELIMINATION OF PEMPIDINE IN THE RAT

BY

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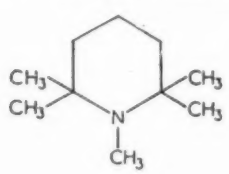
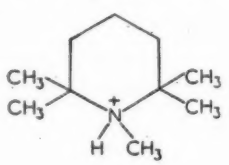
Pempidine (1,2,2,6,6-pentamethylpiperidine) is a ganglion blocking agent introduced recently for the treatment of hypertension by oral administration of its hydrogen tartrate. It can be estimated colorimetrically by coupling with methyl orange, or fluorimetrically by reaction with eosin in xylene, the limits of sensitivity being 0.5 μg./ml. and 0.001 μg./ml. respectively. These methods, combined with appropriate extraction techniques, were suitable for estimating pempidine in aqueous solutions of its salts, in biological fluids and the like, and for investigating the biochemical properties of the drug when given orally to rats in amounts similar to those used clinically.

When administered orally to rats pempidine was rapidly absorbed, the maximum concentration in plasma being attained after 30 min. The drug was preferentially taken up by erythrocytes and a red cell/plasma partition ratio of about 1.2 established with clinical doses. Pempidine was soon distributed throughout the body, including the cerebrospinal fluid, and the highest concentrations were found in kidney, spleen and liver. Pempidine also entered the foetus and passed thence into the amniotic fluid. Protein-binding of the drug occurred only to a very limited extent and there was little evidence that it was metabolized. Pempidine was excreted rapidly in urine during 24 hr. following oral administration.

Pempidine (1,2,2,6,6-pentamethylpiperidine) is well absorbed when given by mouth. It is a tertiary heterocyclic amine (I) and a strong base ($pK_a \sim 11$) which forms a water-soluble hydrochloride and hydrogen tartrate. Under physiological conditions ($pH < 8$) it exists almost entirely (more than 99.9%) in the ionized form (II). The pharmacological properties of this



I



II

compound were first reported by Spinks and Young (1958) and independently by Lee, Wragg, Corne, Edge, and Reading (1958) and by Corne and Edge (1958). The clinical studies by Harington, Kincaid-Smith, and Milne (1958) suggested that pempidine had important advantages in the treatment of hypertension since the drug is freely absorbed after oral administration and is rapidly excreted in the urine.

METHODS

Estimation

Methyl Orange Complex.—Brodie and Udenfriend (1945) estimated various alkaloids and synthetic basic organic compounds in biological material by coupling with methyl orange. This technique was employed by Baer, Paulson, Russo, and Beyer (1956) and again by Allanby and Trounce (1957) in work on mecamylamine, and has now been successfully applied to the estimation of pempidine in biological fluids and tissue extracts.

A master calibration was obtained by adding known amounts of pempidine to ethylene dichloride and treating with the methyl orange reagent. The plot of optical density at 540 mμ against concentration of pempidine was linear up to 50 μg./ml., the lower limit of sensitivity being about 0.5 μg./ml. Standard curves for pempidine in water, urine, plasma, haemolysed red cells, and various tissue homogenates were prepared by adding measured quantities of the hydrogen tartrate to 1 ml. samples of those fluids, making 2 M with respect to sodium hydroxide and extracting with 20 ml. of ethylene dichloride. Methyl orange was added to this extract and the optical density measured at 540 mμ. After appropriate blank corrections, the optical density was a linear function of drug concentration and comparison of these plots with the master

calibration showed that extraction of pempidine from aqueous media into ethylene dichloride was virtually complete (>93%). The technique was rather insensitive but could be used to measure the amount of pempidine in urine from rats which have received the drug orally. However, relatively large doses (>20 mg. base/kg.) were required to produce measurable concentrations of the drug in plasma and tissues.

Eosin Fluorescence.—The methyl orange method could not detect the very low plasma concentrations of pempidine attained after oral administration of the drug in amounts which were effective clinically (0.25 to 0.50 mg. base/kg.) and a more sensitive method of estimation was therefore required. Use has been made of the fact that eosin (tetrabromofluorescein) dissolved in xylene exists in a non-ionized form (HA) which is non-fluorescent. Addition of pempidine (B) produces a proportional change in the reagent to an ionized form which is intensely fluorescent: $HA+B \rightarrow (\overset{+}{B}H)(\bar{A})$. The intensity of fluorescence was proportional to the pempidine concentration, and the technique was extremely sensitive, 0.001 μg./ml. being detectable with the relatively simple equipment shown diagrammatically in Fig. 1. The source of radiation was a 125 watt mercury vapour lamp, **L** (Siemens type MB/D), fed from the constant voltage transformer (**T**). Exciting light of wavelength 545 mμ, isolated by the primary filter $\mathbf{F_1}$ (Ilford type 807), passed through the collimator (**C**) to the fused glass sample cell (**S**). Fluorescence radiation of wavelength 570 mμ emitted by the sample passed through the secondary filter $\mathbf{F_2}$ (Ilford type 808) which absorbs scattered exciting radiation, on to the photomultiplier **P** (RCA type 931 A) connected to the sensitive galvanometer **G** (Cambridge type No. 41127). A stabilized power supply (**V**) was used to operate the photomultiplier. An opaque shutter (**D**) was introduced into the exciting beam when the detector dark current is measured.

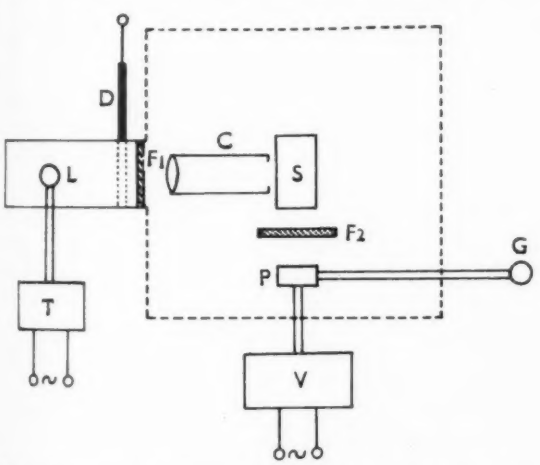


FIG. 1.—Fluorescence apparatus. (**L**), 125 watt mercury vapour lamp (Siemens type MB/D). (**T**), Constant voltage transformer. (F_1), Ilford type 807 filter for the selection of exciting light of wavelength 545 mμ. (**C**), Collimator. (**S**), Fused glass sample cell. (F_2), Ilford type 808 filter for the absorption of scattered exciting radiation. (**P**), Photomultiplier (R.C.A. type 931 A). (**G**), Sensitive galvanometer (Cambridge type No. 41127). (**V**), Stabilized power-pack to operate the photomultiplier. (**D**), Opaque shutter.

Eosin was prepared by adding hydrochloric acid to an aqueous solution of the sodium salt (B.D.H.), washing the precipitate until free from acid and drying *in vacuo*. 0.5 g. of the solid was shaken for 1 hr. with 100 ml. of xylene, and the saturated solution filtered. If stored in the dark, the reagent was quite stable for at least a month.

A master calibration was obtained by using suitably diluted standard solutions of pempidine in xylene (15 ml.) and eosin reagent (2 ml.). The plot of fluorescence intensity against concentration of pempidine was linear up to 0.3 μg./ml., the lower limit of sensitivity being approximately 0.001 μg./ml. Standard curves for pempidine in water, plasma, haemolysed red cells, and various tissue homogenates were prepared by adding measured quantities of the hydrochloride to 5 ml. samples of these fluids which were then made 2 M in sodium hydroxide and extracted with ether (15 ml.). The base was taken into M-hydrochloric acid (2 ml.) which in turn was made 2 M in sodium hydroxide, re-extracted with xylene (15 ml.) and finally transferred to the sample cell. Appropriate blank corrections were applied and the fluorescence intensity shown to be a linear function of concentration. Extraction of pempidine from aqueous media by this method was about 75% complete and quite reproducible. The technique cannot at present be employed with urine, which contains interfering substances able to quench fluorescence. Aqueous solutions of pempidine hydrochloride were examined by both methyl orange complex and eosin fluorescence techniques and gave results which agreed within the limits of experimental error. The eosin fluorescence method seemed to offer great possibilities in the field of ultra-micro analysis and full details will be published elsewhere.

Preparation of Samples for Analysis

In most of the experiments described below, pempidine was administered as the hydrogen tartrate, but the hydrochloride was used in experiments designed to investigate its passage into the cerebrospinal fluid. Mecamylamine was used as the hydrochloride. All doses and concentrations of the two drugs are given as base equivalents.

Drug Concentrations in Blood.—Single oral doses of pempidine within the range 0.25 to 20 mg./kg. were administered in aqueous solution by stomach tube to albino rats (100 to 150 g.) which had been deprived of food for 16 hr. The animals were killed at intervals during the ensuing 24 hr. and heparin added to blood obtained by heart puncture. After centrifuging, the volumes of plasma and erythrocytes in pooled samples from two or three rats were

measured and the concentration of pempidine in each estimated by the eosin fluorescence method, the red cells being first diluted with distilled water equal in volume to the plasma removed.

Tissue Distribution.—Pempidine (1 or 10 mg./kg.) was given orally to rats which were killed and exsanguinated at known time intervals after dosage, the various organs then being removed and homogenized in distilled water. Concentrations of the drug in foetus and amniotic fluid were investigated using pregnant rats after oral dosage at 10 mg./kg. Estimations were carried out with eosin (low dose) or methyl orange (high dose) on pooled samples from three or five animals, except with foetal material where only two rats could be used.

Penetration into Cerebrospinal Fluid.—Pempidine (5 mg./kg.) as the hydrochloride in normal saline was injected intravenously into adult rabbits which were killed with pentobarbitone after a measured time interval; samples of cerebrospinal fluid and blood were withdrawn by cisternal and heart puncture respectively. Each of these experiments was done on at least two animals. Similar experiments were carried out with mecamylamine.

Urinary Excretion. — Single oral doses of pempidine (2.5 to 20 mg./kg.) as the hydrogen tartrate were administered to albino rats (100 to 150 g.) which were kept in metabolism cages with free access to water. Twenty-four hour collections of urine were made for three days after dosage, estimations being performed with methyl orange on pooled samples from three animals.

The effect of urinary *p*H on excretion of pempidine and mecamylamine was also studied. Rats in groups of 12 were given N/10-sodium bicarbonate, N/10-ammonium chloride or water to produce alkaline, acid or normal urine before and after administering a single oral dose of pempidine (10 mg./kg.) as hydrogen tartrate, or mecamylamine (10 mg./kg.) as hydrochloride. Thirty-six animals were used for each compound, the urine being collected at hourly intervals.

Methyl orange, however, was not specific for pempidine and any basic metabolites of the drug would also be estimated by this method. All bases present in the ethylene dichloride extract of urine were therefore removed with hydrochloric acid which was then evaporated to dryness for further investigation. That the material extracted from urine was a single substance indistinguishable from pempidine was suggested by (*a*) paper chromatography in the organic phase of the system butanol/acetic acid/water = 100/10/100 (v/v), (*b*) paper electrophoresis at *p*H 7, using phosphomolybdic acid to locate the spots according to Chargaff, Levine, and Green (1948), and (*c*) by studies of partition between ethylene dichloride and aqueous buffer solutions at *p*H values between 5.0 and 10.7. Infra-red spectroscopy finally established that the excreted compound was, in fact, pempidine.

In Vitro Experiments

Protein-binding of Pempidine.—The binding of pempidine to plasma proteins was studied by dialysis. Solutions of pempidine (250 μg./ml.) in horse plasma were dialysed in Visking tubing at room temperature against physiological saline. The drug concentrations inside and outside the dialysis sac were estimated with methyl orange until equilibrium was established after 72 hr. Preliminary experiments showed that the membrane was completely permeable to pempidine but retained proteins.

RESULTS

Drug Concentrations in Plasma and Erythrocytes.—Fig. 2 shows the concentrations of pempidine in plasma at various times after a single oral dose of the hydrogen tartrate. The curves are typical of those obtained at all doses within the range 0.25 to 20 mg./kg. At first the drug concentration increased rapidly, and in 30 min. attained a maximum value proportional to the amount of drug administered (Fig. 3). At doses not exceeding 5 mg./kg., the plasma concentration, having passed its maximum, decreased quite quickly but after 2 hr. fell more slowly so that appreciable concentrations of drug were maintained for 6 hr. or more. With larger doses, a second maximum in the plasma concentration appeared at 4 to 6 hr.; this phenomenon occurred regularly and was not attributable to error of measurement, but its significance has not been studied further.

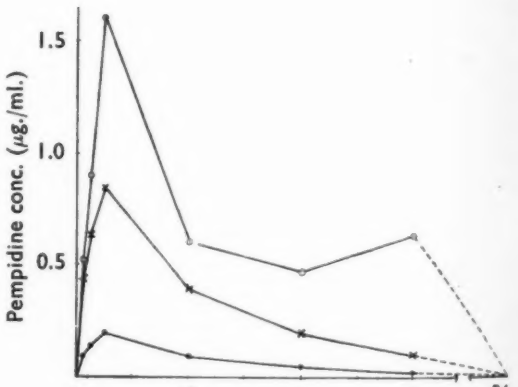


FIG. 2.—Plasma concentrations of pempidine at various times after the administration of single oral doses to rats. The compound was given as an aqueous solution of the hydrogen tartrate. Each point represents an estimation on pooled heart-blood from three animals. The plasma concentrations were measured after 10 mg./kg. (upper curve), 5 mg./kg. (middle curve) and 1 mg./kg. (lower curve).

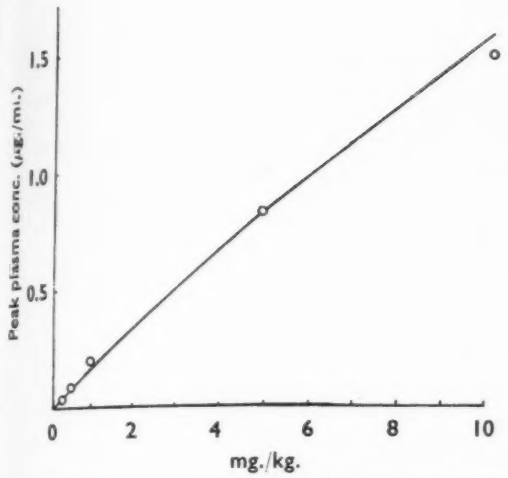


FIG. 3.—Peak plasma concentration of pempidine after various single oral doses to rats. The compound was given as an aqueous solution of the hydrogen tartrate. Each point represents an estimation on pooled heart-blood from three animals 30 min. after receiving the dose.

After a single oral dose of pempidine (1 mg./kg.) as the hydrogen tartrate, the drug concentration in erythrocytes rapidly increased during the first 30 min., and thereafter decreased in a manner closely resembling that observed with plasma (Table I). This indicated that the drug passed rapidly into (or on to) the erythrocytes, a partition ratio of about 1.2 being maintained in favour of the cells. When pempidine was administered in lethal amounts (175 mg./kg. of pempidine base injected intraperitoneally) the partition ratio was lowered to 1.03.

Tissue Distribution of Pempidine.—Concentrations of drug found in tissues from rats dosed orally with pempidine (1 mg./kg. or 10 mg./kg.) as the hydrogen tartrate and killed 1 or 4 hr. later are given in Tables II and III, which also show the corresponding tissue/plasma ratios.

TABLE I

DISTRIBUTION OF PEMPIDINE BETWEEN ERYTHROCYTES AND PLASMA

Pempidine (1 mg./kg.) was administered orally to rats as an aqueous solution of the hydrogen tartrate and each estimation performed on pooled heart-blood from three animals. Estimations by the eosin fluorescence method.

Time After Dose (min.)	Plasma Conc. (μg./ml.)	Erythrocyte Conc. (μg./g.)	Erythrocyte/ Plasma Conc. Ratio
10	0·110	0·128	1·17
20	0·175	0·204	1·17
40	0·165	0·194	1·18
80	0·120	0·138	1·17
160	0·050	0·058	1·21
360	0·020	0·023	1·15

TABLE II

DISTRIBUTION OF PEMPIDINE IN TISSUES (1 MG./KG.)

Pempidine (1 mg./kg.) was administered orally to rats as an aqueous solution of the hydrogen tartrate. Estimations were carried out on tissues obtained from three rats which were killed 1 hr. after receiving the drug. Estimations by eosin fluorescence method.

Tissue	Drug Conc. (μg./g.)	Tissue/Plasma Conc. Ratio
Brain	0·28	2·16
Erythrocytes	0·154	1·19
Kidney	1·77	13·6
Liver	1·25	9·6
Lung	0·36	2·77
Voluntary muscle	0·22	1·54
Plasma	0·130	—
Spleen	1·35	10·4

TABLE III

DISTRIBUTION OF PEMPIDINE IN TISSUES (10 MG./KG.)

Pempidine (10 mg./kg.) was administered orally to rats as an aqueous solution of the hydrogen tartrate. The organs were removed from five rats either 1 or 4 hr. after receiving the drug. Two rats were used for drug estimations in foetal tissue and amniotic fluid. Estimations by methyl orange method, which, when applied to tissues, tends to give high blanks.

Tissue	Drug Conc. (μg./g.)		Tissue/Plasma Conc. Ratio	
	1 hr.	4 hr.	1 hr.	4 hr.
Brain	2·1	Nil	0·5	—
Kidney	85·0	8·1	21·0	4·0
Liver	9·5	7·8	2·0	3·9
Lung	13·5	5·7	3·4	2·9
Voluntary muscle	4·5	Nil	1·1	—
Spleen	16·3	32·3	4·1	16·2
Foetus	24·3	8·8	8·1	4·4
Amniotic fluid	3·0	3·7	0·8	1·9
Plasma	4·0	2·0	—	—

After 1 hr., the highest concentrations of pempidine were found in kidney and spleen with rather smaller amounts in liver. 2 and 4 hr. after administration of the drug, the amount of pempidine in the various tissues had fallen except in the spleen where the drug concentration had increased. Only very small amounts of drug could be detected in both spleen and liver up to 72 hr.

With pregnant rats appreciable concentrations of pempidine were observed in the foetus 1 hr. after giving the drug orally. At the end of 4 hr., however, much of the pempidine had passed into the amniotic fluid.

Penetration into C.S.F.—Fig. 4 shows curves for the concentration of pempidine and mecamylamine in cerebrospinal fluid during the six hours following a single intravenous injection of the hydrochlorides. Each point represents the drug concentration in cerebrospinal fluid expressed as a percentage of the corresponding plasma concentration. Pempidine and mecamylamine entered the cerebrospinal fluid to approximately the same extent, concentrations of

FIG. 4.—Penetration of pempidine and mecamylamine into the cerebrospinal fluid of rabbits following a single intravenous injection of 5·0 mg./kg. of one or other as the hydrochloride in saline.

nearly 50% being attained during the first hour. The concentration of mecamylamine in cerebrospinal fluid relative to plasma remained high for 6 hr., whereas that of pempidine fell during this period. Since the plasma concentrations and the plasma/erythrocyte partition ratios of the two drugs were similar, the absolute rate of elimination of pempidine from the cerebrospinal fluid was more rapid than that of mecamylamine.

Urinary Excretion.—The urinary excretion of pempidine is shown in Fig. 5. Most of the drug was excreted during the first 24 hr., only 2% being found in the second 24 hr. and negligible quantities thereafter. In these experiments the *p*H of the urine (~6.9) was not significantly different from untreated controls. The total recovery of drug varied with the dose, 40 to 45% in the lower ranges, 70 to 80% with larger amounts. Under all conditions of urinary *p*H, the excretion of pempidine was more rapid and complete than that of mecamylamine. Fig. 6 shows the excretion in acid (*p*H 5.6) and alkaline (*p*H 8.2) urine of the two drugs after a single oral dose. Only very small amounts (less than 0.1% of the dose) of pempidine were found in faeces during the 48 hr. following a single oral dose of the drug.

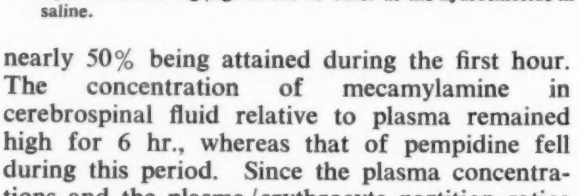


FIG. 5.—Cumulative urinary excretion of pempidine, expressed as % recovery from urine, in rats following single oral doses of aqueous solutions of the hydrogen tartrate. 10 and 20 mg./kg. (upper curve), 2·5 mg./kg. (middle curve), 5·0 mg./kg. lower curve.

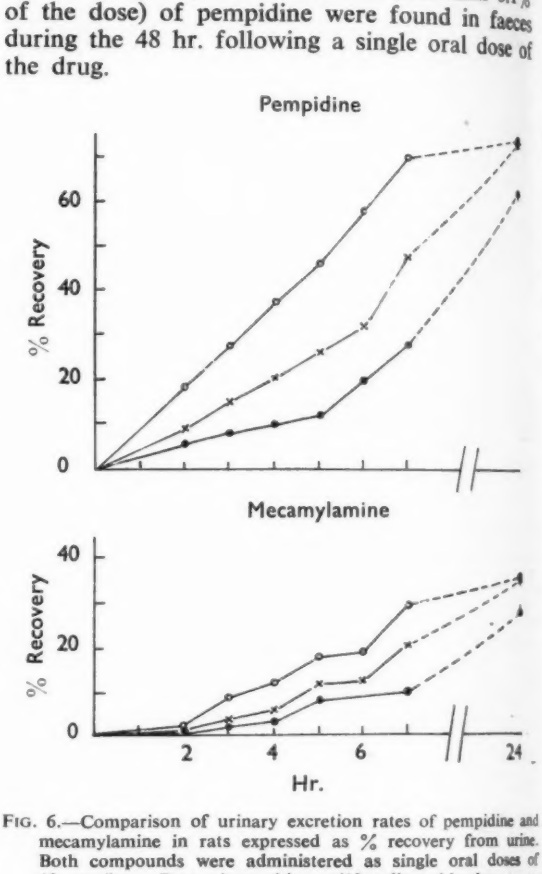


FIG. 6.—Comparison of urinary excretion rates of pempidine and mecamylamine in rats expressed as % recovery from urine. Both compounds were administered as single oral doses of 10 mg./kg. Rats given either N/10-sodium bicarbonate or N/10-ammonium chloride as drinking water to induce excretion of an acid or alkaline urine respectively. Recovery from acid urine, upper curve in each graph; from normal urine, middle curves; and from alkaline urines, lower curves.

Protein-binding of Pempidine.—Binding of pempidine to plasma proteins only occurred to a very limited extent. An average value of 1.2% (limits 0.5 to 2.6%) was obtained in a number of experiments for the proportion of drug bound to protein, the estimations being carried out with varying doses of drug.

DISCUSSION

The methyl orange complex and eosin fluorescence techniques were both satisfactory for estimating pempidine in certain body fluids and may be regarded as complementary. Methyl

orange, with rather limited sensitivity, can be used to study excretion with small doses because the concentration of the drug in urine is relatively high, but measurements of absorption and tissue distribution are possible only if large amounts of pempidine have been administered. If the dose was less than 20 mg./kg. the methyl orange method was unable to detect the very low concentrations of pempidine in plasma and tissues and was too insensitive for use in clinical investigations (Harington *et al.*, 1958). The eosin fluorescence method, on the other hand, is eminently suitable for plasma and tissue samples after oral doses of pempidine in the clinical range but cannot at present be applied to urine because of interfering substances which quench the fluorescence.

When given orally at doses within the range 1 to 10 mg./kg., pempidine was very rapidly absorbed and the peak plasma concentration, attained in about 30 min., was proportional to the dose. The amount in plasma was small when compared with the dose, and this suggested that it was widely distributed. The concentration of pempidine in erythrocytes was significantly higher than in plasma, a partition ratio of ~1.2 being quickly established and then maintained for some hours. After lethal intraperitoneal injections of pempidine (doses of about 200 mg./kg.) we found a lower value (1.03) in agreement with the findings of Harington *et al.* (1958). With these large doses the red cells probably became saturated with pempidine, and the concentration was thus at a constant and maximum level, whilst the amount of drug in the plasma rose and hence lowered the partition ratio. Milne, Rowe, Somers, Muehrcke, and Crawford (1957) reported a similar distribution of mecamylamine between erythrocytes and plasma (partition ratio ~1.15) and showed that it was a function of the *p*H of the extracellular fluid and therefore possibly depended upon the relative concentrations of ionized and unionized drug present. The effect is unlikely to be a consequence of protein binding, for although the partition ratios for mecamylamine and pempidine are similar their protein-binding properties are very different. After adding mecamylamine to plasma *in vitro*, Milne found 25% of the drug was bound to protein, whereas our experiments under similar conditions showed that protein binding of pempidine occurred to only an insignificant extent.

Pempidine, administered orally, rapidly became distributed throughout the body tissues, and, 1 hr. after dosing, the highest concentrations were found in the kidney, spleen, and liver. After 4 hr., the drug was concentrated mainly in the spleen, presumably owing to a rapid turn-over of red cells. The placenta afforded no barrier to pempidine, which readily penetrated into foetal tissue. Four hours after oral administration, the concentration of drug in the foetus had fallen whilst the amount in the amniotic fluid had increased, indicating that the foetus excreted pempidine.

Pempidine and mecamylamine were both found in the cerebrospinal fluid, as was inferred by Corne and Edge (1958), who noted the appearance of tremors when large doses of pempidine or mecamylamine were given orally or parenterally. Brodie and Hogben (1957) postulated that the rate at which drugs pass into or out of the central nervous system depends on the lipoid solubility of the uncharged molecules. But other factors must also be involved, such as the extent to which protein binding occurs and particularly the proportion of base cations and uncharged molecules present at physiological *p*H. Thus mecamylamine and pempidine are almost completely ionized at *p*H 7.2 and have similar low partition coefficients between ethylene dichloride and aqueous buffer at that *p*H [Brodie and Hogben (1957) used partition between chloroform and water as the criterion of lipoid solubility in this context]. The fact that neither drug attained a concentration in cerebrospinal fluid equal to the plasma level even 3 hr. after administration is in accord with the views of Brodie and Hogben (1957). However, while the two drugs entered the cerebrospinal fluid of rabbits at about the same rate, pempidine disappeared more rapidly than mecamylamine. Whatever the reason, it might be expected that any central nervous effects which pempidine might have in man may be of shorter duration and less severe than those seen with mecamylamine.

Pempidine was excreted rapidly in urine during the 24 hr. following oral administration, the rate of excretion in the first 7 hr. being almost twice that of mecamylamine. At doses approaching those used in clinical practice, 45% of the drug appeared in the urine within 24 hr. (70 to 80% with larger doses), but very little of the drug was recovered subsequently. We have no evidence of the fate of the remainder of the dose administered. Renal excretion of both pempidine and mecamylamine is affected by urinary *p*H. Thus 75% and 63% recoveries of administered drug are obtained with pempidine under acid and alkaline conditions compared with 36% and 28%

respectively for mecamylamine. Mecamylamine was used as a reference compound for these studies because it is, we believe, the only non-quaternary ganglion blocking agent which is in clinical use. Differences in behaviour are, however, not wholly unexpected since mecamylamine is a secondary and pempidine a tertiary amine.

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THE EFFECT OF MORPHINE ON VAGAL INHIBITION OF THE HEART

BY

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The action of morphine (0.1 to 100 mg./kg.) on the cardiac slowing produced by stimulation of the right vagus nerve varied in the four species investigated. In the guinea-pig, morphine had no inhibitory effect and in the cat the effect was small, but in the rat and the rabbit stimulation of the vagus slowed the heart much less after administration of morphine than before. Further, in the last two species morphine delayed the onset of cardiac slowing. Nalorphine, which given alone had a slight morphine-like action, partly reversed the effect of morphine.

Small concentrations of morphine are known to inhibit the muscular contractions involved in both the preparatory and emptying phases of the peristaltic reflex in the guinea-pig ileum (Trendelenburg, 1917; Schaumann, 1955; Kosterlitz and Robinson, 1955, 1957). It seemed, however, that a detailed analysis of the peripheral effects of morphine might be easier in a preparation in which the anatomical arrangement of nervous structures was simpler than that in gut and also that it would be useful to study what happened when such nervous pathways mediated an inhibitory rather than an excitatory effect. For these reasons the inhibition of the sinoauricular node by vagal stimulation was chosen.

METHODS

Four species were used, namely, the cat, guinea-pig, rat, and rabbit. Cats were anaesthetized with ether and spinal preparations made by a modified Dale technique (Kosterlitz, Krayer, and Matallana, 1955); at least 1 hr. elapsed before the start of the experiment. All other animals were anaesthetized with a solution of 10% urethane and 1% chloralose (w/v) injected intraperitoneally. Rats were given 7.5 ml./kg. and the other animals 5 ml./kg.

The trachea was cannulated and, except in the rats, artificial respiration applied. In the cats, both stellate ganglia were removed through the first intercostal space. The vagus nerve on the left side was cut; that on the right side was exposed as far as the ganglion nodosum, and care was taken to keep the blood supply to the nerve intact. The area was then flooded with warm liquid paraffin. The nerve was ligated and cut, and the distal end was placed on the stimulating electrodes. It was stimulated with rectangular pulses from a Grass stimulator, using a RF-coupled output stage for isolating the stimuli from earth. The pulse duration was 0.1 msec. and the voltage was selected to give a supramaximal stimulus; this voltage was then maintained throughout the experiment. In experiments in which the vagus was stimulated at one frequency only, this was so chosen as to reduce the unstimulated or resting heart rate by about one third. When stimulus frequency/response curves were constructed, frequencies high enough to produce rhythms other than sinoauricular were excluded.

Recording.—The blood pressure in the left femoral artery was measured with a condenser manometer and recorded with the electrocardiogram by a pen oscillograph.

The resting heart rate was counted from the electrocardiogram for a period of 10 sec. at the beginning of each successive minute, except immediately after a period of stimulation. The vagus nerve was stimulated during every third minute for 30 sec. or every fourth minute for 60 sec., and the heart rate was counted from the 15th to the 25th sec. after the beginning of stimulation; when the stimulation lasted for 60 sec. a second count was taken from the 45th to the 55th sec. The heart rate was plotted against time (Fig. 1). To facilitate interpretation of the results, the average resting heart rate (the mean of the two counts immediately before stimulation) and the maximum fall in rate during stimulation were each plotted against time (Figs. 2 and 3). When stimulus frequency/response curves were determined, the fall in rate was plotted as % of the resting heart rate against the logarithm of the stimulus frequency. Before the administration of morphine two superimposable control curves were obtained. Then morphine was injected and the series of observations was repeated.

Drugs.—All injections of morphine hydrochloride or nalorphine hydrobromide were made into the right femoral vein; the doses, which varied from 0.05 to 100 mg./kg., refer to the salts in aqueous solution.

RESULTS

Cat.—In 8 experiments on cats, the stimulus frequency varied between 0.3 and 3/sec. but was kept constant throughout any one experiment. Morphine in doses of 0.1 to 10 mg./kg. had no lasting effect on the resting heart rate; in some preparations the larger doses (5 to 10 mg./kg.) caused a transient rise in heart rate and arterial blood pressure which was assumed to be due to a liberation of catechol amines (Fig. 1). In most experiments after injection of morphine, there was a reduction in the cardiac slowing on stimulation of the vagus (Fig. 1); this, however, was small and, on the average, a dose of 1 mg./kg. reduced the effect by 18%.

Guinea-pig.—In 5 experiments on guinea-pigs there was no evidence that morphine, in doses of 0.1 to 5 mg./kg., reduced the cardiac slowing caused by vagal stimulation. A difficulty in some of these experiments was a tendency for the resting rate to rise after injection of morphine; nevertheless, when this occurred there was no diminution of the vagal effect. In the experiment shown in Fig. 2*a* the resting rate did not change significantly and the effect of vagal stimulation was, if anything, enhanced by morphine.

Rat.—Twelve experiments were performed on rats. After morphine, in doses of 0.1 to 2.5 mg./kg., there was an immediate sharp decrease in the degree of slowing produced by stimulating the vagus at a given frequency (3 to 15/sec.) (Fig. 2*b*). Although in some experiments recovery was fairly rapid and complete, in others it was not apparent within 1 hr. That this was not necessarily due to a failure of the nerve was clear from the fact that the morphine-antagonist, nalorphine, produced at least partial recovery an hour or more after the injection of morphine in some, though not in all, experiments.

Since morphine in doses larger than 1 mg./kg. depressed respiration severely, a small dose of nalorphine was given before the injection of the

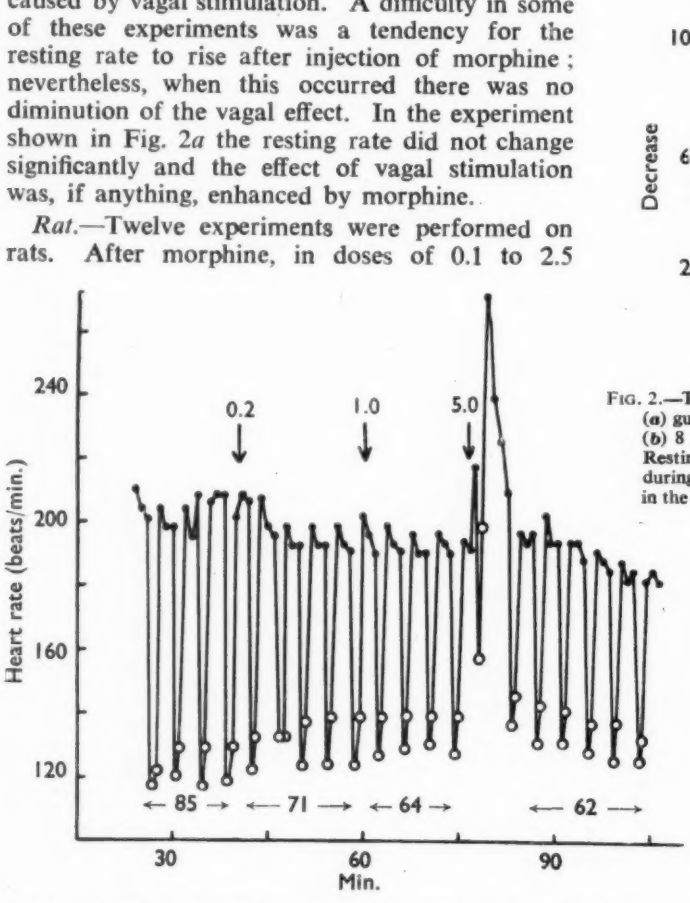


FIG. 1.—The effect of morphine on vagal inhibition of the heart in the cat. Spinal preparation, stellate ganglia removed. ●——●, resting heart rate; O——O, heart rate during vagal stimulation (12 V., 0.1 msec., 3/sec., every 4 min. for 60 sec.). At the arrows, morphine was injected in the doses indicated (mg./kg.). The numerals below the tracing give the average slowing (beats/min.) produced by vagal stimulation.

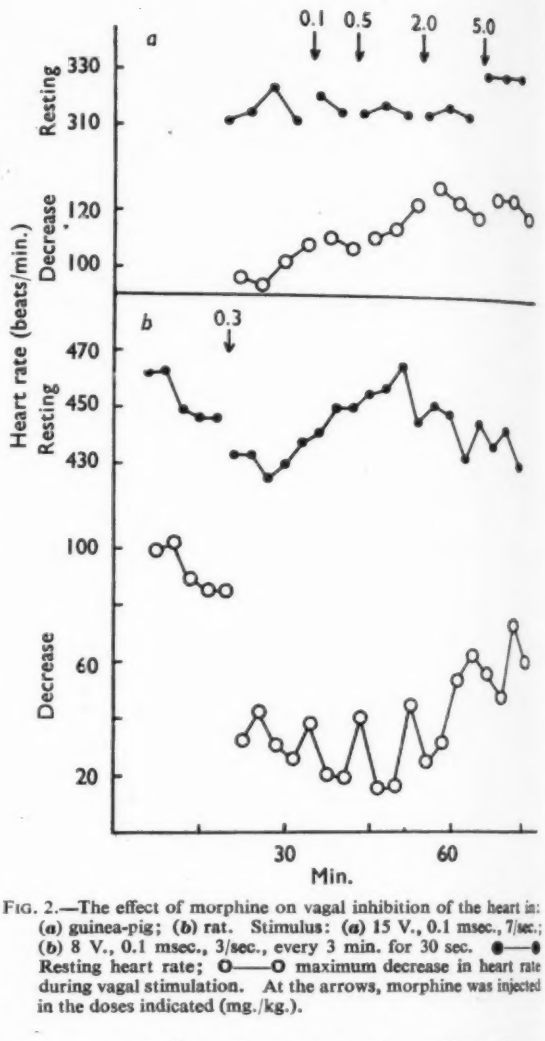


FIG. 2.—The effect of morphine on vagal inhibition of the heart in: (*a*) guinea-pig; (*b*) rat. Stimulus: (*a*) 15 V., 0.1 msec., 7/sec.; (*b*) 8 V., 0.1 msec., 3/sec., every 3 min. for 30 sec. ●——● Resting heart rate; O——O maximum decrease in heart rate during vagal stimulation. At the arrows, morphine was injected in the doses indicated (mg./kg.).

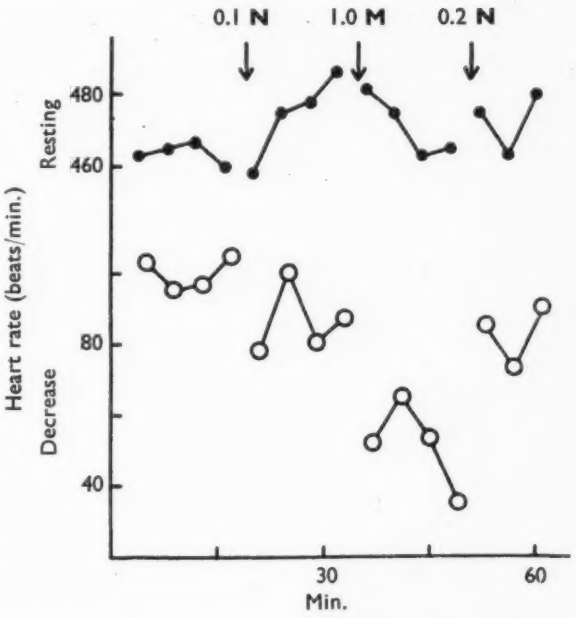


FIG. 3.—The effects of nalorphine and morphine on vagal inhibition of the heart in the rat. Stimulus: 8 V., 0.1 msec., 6/sec., every 3 min. for 30 sec. ●——● Resting heart rate; O——O maximum decrease in heart rate during vagal stimulation. At the arrows, nalorphine (N) or morphine (M) was injected in the doses indicated (mg./kg.).

larger doses of morphine. In 2 of these experiments, nalorphine had no effect on the response to vagal stimulation, whereas in 3 others a morphine-like effect was noted. After a preliminary injection of nalorphine (Fig. 3), morphine, in doses up to twice that of the nalorphine, did not inhibit vagal slowing; but larger doses of morphine did, although respiration remained relatively unaffected.

It was observed that, quite apart from the effect of morphine on the degree of slowing, the onset of this was always markedly delayed. When the vagus was stimulated at 40/sec., morphine scarcely affected the degree of slowing, its only action being to produce a delay in onset (Fig. 4).

Rabbit.—In 4 out of 20 experiments on rabbits the method of assessing the results was the same as in the other species. The effects of morphine and nalorphine were similar to those obtained in the rat.

In the course of these experiments we noticed that, in the rabbit, the resting heart rate remained constant much longer than in the rat and that the vagal responses were reproducible for long periods. For these reasons we chose the rabbit for a more detailed investigation of the stimulus frequency/response curves. The degree of cardiac slowing, expressed as % of the resting heart rate, was plotted against the stimulus frequency, which

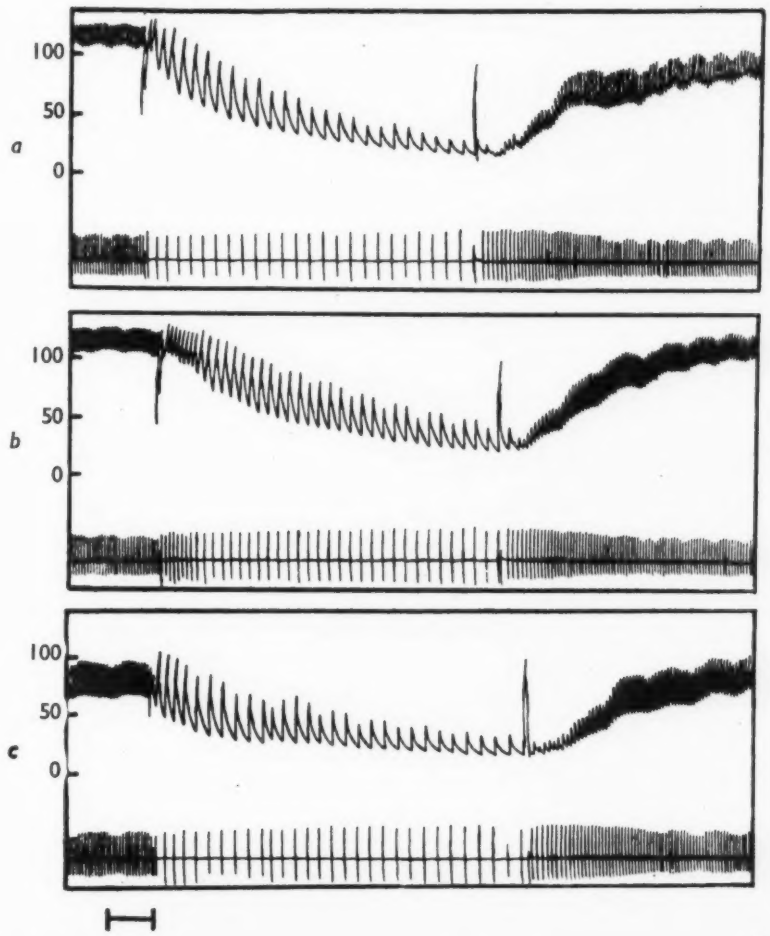


FIG. 4.—The delay caused by morphine on the onset of cardiac slowing after vagal stimulation. Rat. Stimulus: 8 V., 0.1 msec., 40/sec., between signals. (*a*) control. Between (*a*) and (*b*) 0.1 mg./kg. nalorphine was injected, without effect on the responses, followed by 1 mg./kg. morphine. Between (*b*) and (*c*) 2 mg./kg. morphine was injected without altering the responses shown in (*b*) followed by 2 doses of nalorphine, 0.2 and 0.5 mg./kg., respectively. Upper of each pair of traces: blood pressure mm. Hg; lower: electrocardiogram. Time: 2 sec.

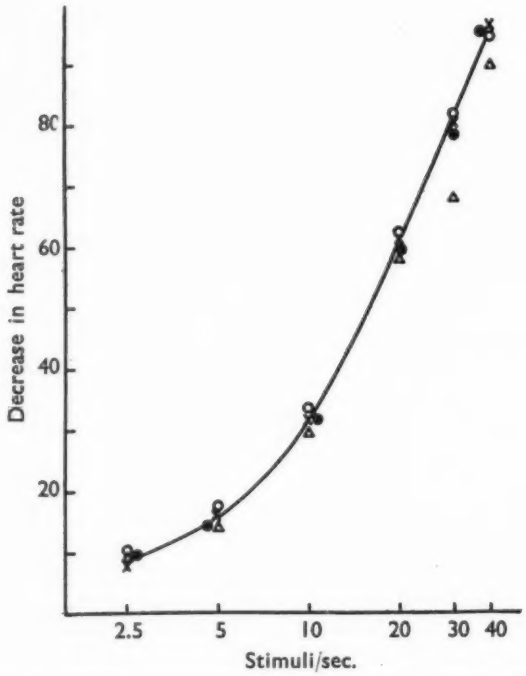


FIG. 5.—Stimulus frequency/response curve showing the effects of vagal stimulation on the heart rate in the rabbit. ●——● At beginning of the experiment; O——O after 48 min.; X——X after 120 min.; Δ——Δ after 187 min. Mean resting heart rates 300, 300, 300, and 326 min., respectively. The vagus was stimulated every 3 min. for 30 sec. Ordinate, decrease in heart rate expressed as % of resting rate.

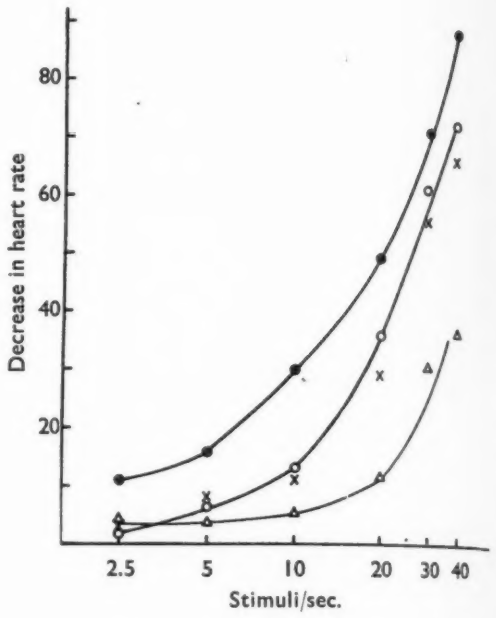
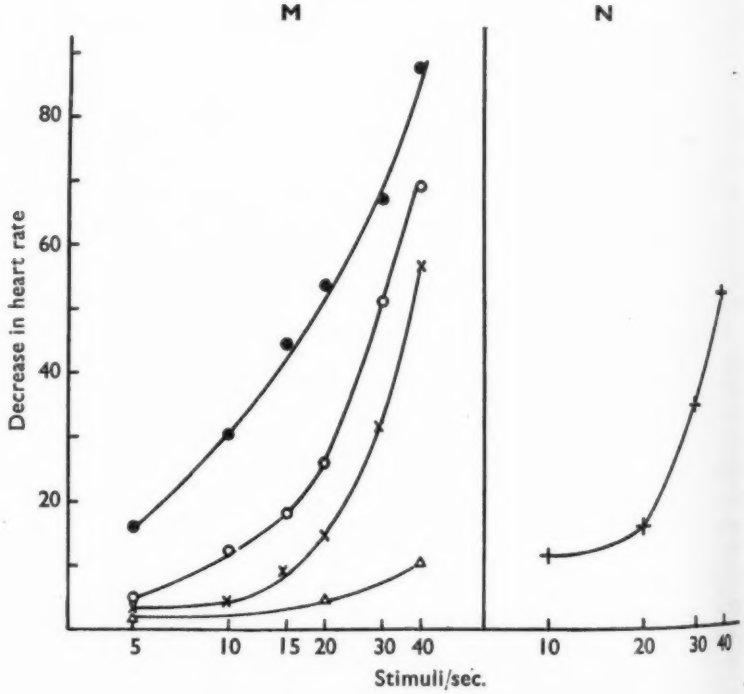


FIG. 7.—The effects of morphine (1 mg./kg.) at different intervals after injection on vagal inhibition of the heart in the rabbit. ●——● Control; O——O mean of values obtained between 18 and 51 min. after injection; X——X 73 to 88 min. after injection; Δ——Δ 164 to 179 min. after injection. Mean resting heart rates 301, 302, 320, and 242 min., respectively. The vagus was stimulated every 3 min. for 30 sec. Ordinate, as in Fig. 5.

FIG. 6.—The effect of morphine (M) and nalorphine (N) on vagal inhibition of the heart in the rabbit. ●——● Control; O——O after 1 mg./kg. morphine; X——X after additional 10 mg./kg. morphine; Δ——Δ after additional 100 mg./kg. morphine; +——+ after additional 24 mg./kg. nalorphine. Mean resting heart rates 292, 293, 276, 251, and 292 min., respectively. The vagus was stimulated every 3 min. for 30 sec. Ordinate, as in Fig. 5.



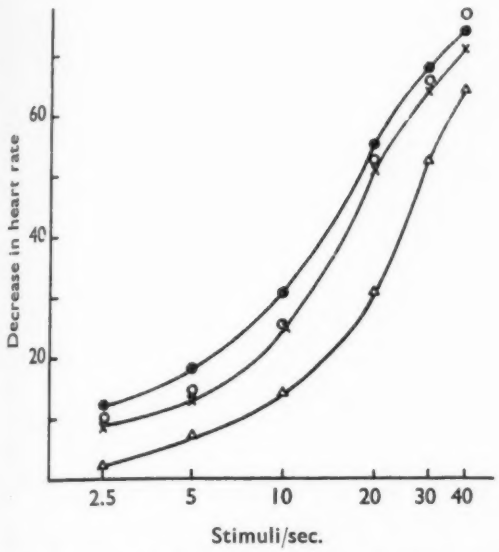


FIG. 8.—The effects of nalorphine followed by morphine on vagal inhibition of the heart in the rabbit. ●——● Control; O——O after 0.5 mg./kg. nalorphine; ×——× after additional 5 mg./kg. nalorphine; Δ——Δ after additional 10 mg./kg. morphine. Mean resting heart rates 298, 296, 292, and 294 min., respectively. The vagus was stimulated every 3 min. for 30 sec. Ordinate, as in Fig. 5.

ranged from 0.5 to 40/sec. Stimulation at 40/sec. often led to complete, or almost complete, inhibition of the heart. When this was not so, higher frequencies were usually not more effective and often caused vagus escape and nodal rhythm. The shape of the response curves was usually concave upwards, with the greatest curvature at the lower frequencies; in a small number of experiments S-shaped curves were obtained. When such stimulus frequency/response curves were repeated at intervals of 1 hr. or so, without the administration of morphine, they were practically identical for at least 3 hr. (Fig. 5). The stimulus frequency required to cause a 50% fall in the resting heart rate lay between 10 and 20/sec., with a mean of 14.6/sec.

Morphine, above the threshold dose of 0.1 mg./kg., depressed the response to vagal stimulation at all frequencies (Fig. 6). The inhibitory action of morphine in doses up to 10 mg./kg. was more marked at the lower than at the higher rates of stimulation. The ease with which these results could be interpreted depended to a great extent on whether or not the resting heart rate remained constant. Doses of morphine up to 1 mg./kg. had no significant effect on the resting heart rate for about 1 hr. after administration. During this period the inhibition of vagal slowing was unchanged. However, it was not unusual for the resting heart rate to slow considerably 2½ to 3 hr. after giving morphine, when the inhibitory effect on vagal slowing became much more pronounced (Fig. 7). With large doses of morphine (100 mg./kg.) this marked fall of the resting heart rate and inhibition of vagal slowing occurred during the first few minutes after administration; both these effects were partly reversed by nalorphine (Fig. 6).

Nalorphine given alone had a slight morphine-like effect which, however, was not enhanced by increasing the dose (Fig. 8). We could not decide from the available evidence whether nalorphine, given first, protected against subsequent doses of morphine.

DISCUSSION

Four species were studied. In the guinea-pig, morphine did not reduce the cardiac slowing caused by vagal stimulation; in the cat the effect was relatively small. In the rat and rabbit, on the other hand, stimulation of the vagus produced much less slowing of the heart after morphine than before, and there was also a distinct delay in the onset of such slowing as did occur. Large doses appeared to have a more complex action than small doses in that they not only diminished the cardiac slowing due to vagal stimulation but also led to a fall in the resting, non-stimulated, heart rate. Nalorphine reversed, at least in part, the effects of small or large doses of morphine.

The inhibitory action of morphine on the effect of vagal stimulation might occur at a number of sites, but lack of experimental evidence must render any attempt at interpretation speculative, especially when one takes into account the species differences shown in this paper and known differences in the responses of various structures within any one animal. Thus in the guinea-pig, the peristaltic reflex was inhibited by morphine whereas the vagal slowing was not; on the other hand, in the rabbit, where cardiac slowing due to vagal stimulation was morphine-sensitive, Schaumann (1955) found that the preparatory phase of the peristaltic reflex in the jejunum was not inhibited by morphine.

No evidence is available as to whether or not morphine affects conduction in the vagus nerves of the rat or the rabbit. However, J. W. Thompson (personal communication) found that morphine (10^{-3}) failed to produce any alteration in the resting or action potentials, or in conduction velocity, in the desheathed sciatic nerve of the frog. As far as ganglionic transmission is concerned, no direct evidence is available for the

rabbit or the rat, but Hebb and Konzett (1950) and Trendelenburg (1957) found that morphine did not block transmission in the superior cervical ganglion of the cat. A third possibility is that the effect is produced at the postganglionic nerve endings either by interfering with the formation, release or destruction of acetylcholine, or by altering the sensitivity of the sinoauricular node to acetylcholine. There is no evidence for an increase in destruction of acetylcholine; on the contrary, morphine reduces the activity of brain and serum cholinesterase (Wikler, 1950). Paton (1956, 1957) and Schaumann (1956) both found that morphine reduces acetylcholine release from the guinea-pig ileum stimulated either electrically or by distension. But there is, as yet, no evidence about what occurs at the vagal endings round the sinoauricular node.

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THE STABILITY OF ENDOGENOUS CORTICOTROPHIN IN RAT BLOOD *IN VITRO*

BY

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Endogenous corticotrophin is stable in blood *in vitro* for periods of up to 1 hr. at 4° and 22°, and in plasma for at least 24 hr. at −15°. Estimates of plasma corticotrophin concentration provide less consistent results than assays done on whole blood. The optimal conditions for storing blood prior to the determination of its corticotrophin concentration are discussed.

There is some confusion in the literature concerning the stability of corticotrophin in blood *in vitro*. Several studies on commercial samples of corticotrophin added to blood have had conflicting results. However, very little information has been published on the stability in blood *in vitro* of the *endogenously* secreted hormone. The present work was done because such information is essential to workers concerned with the estimation of circulating corticotrophin. Experiments are described in which blood samples removed from adrenalectomized and stressed adrenalectomized rats were kept under various conditions and then assayed for corticotrophin content.

METHODS

Albino Wistar rats weighing 120 to 160 g. were used in all the experiments. They were fed on a diet of cubes (diet 41, Lane-Petter and Dyer, 1952) and water, and were kept in a room at a constant temperature of 22° for at least one week before use. Approximately 200 female rats were used to provide the blood samples and 270 males for the corticotrophin assays.

Female rats were adrenalectomized bilaterally under ether anaesthesia and were maintained subsequently on the normal diet with 0.9% sodium chloride solution in place of drinking water. Blood was obtained 30 days after adrenalectomy from conscious rats or from animals which had been anaesthetized with ether 2.5 min. previously. The animals were decapitated and blood was collected from their trunks into tubes containing heparin. When plasma samples were used, the plasma was separated after centrifugation of the blood at 3,000 rev./min. for 15 min. Approximately one half of each pooled blood or plasma sample (control) was immediately injected intravenously into groups of at least 6 male rats which had been pretreated with hydrocortisone (Hodges and Vernikos, 1958; Cox, Hodges, and Vernikos, 1958). The remaining half (test) was kept in pyrex glass containers at 4° or 22° for times varying from 40 to 320 min. (whole blood), or at −15° for 24 hr. (plasma), before being similarly injected into hydrocortisone treated rats. A third group of assay rats received injections of normal saline only. All the injections were administered in doses of 3 ml./100 g. body weight. The injected animals were killed 1 hr. later and their adrenal ascorbic acid contents were determined by the method of Roe and Kuether (1943). The mean adrenal ascorbic acid in each group of rats which had received the blood or plasma samples was subtracted from the mean of the corresponding saline injected group. The adrenal ascorbic acid depletion produced by each sample was taken as a measure of its corticotrophin content.

RESULTS

The mean adrenal ascorbic acid depletions produced by blood and plasma samples from adrenalectomized and stressed (ether 2.5 min.) adrenalectomized rats before and after storage for various times at −15°, 4°, and 22° are shown in Table I. The fall in adrenal ascorbic acid induced by the control samples of blood, obtained from adrenalectomized rats and injected immediately after collection, varied between 70 ± 11 and 90 ± 12 mg./100 g. adrenal tissue (mean ± S.E.). None of these results differed significantly ($P<0.05$) from one another. Blood samples from adrenalectomized rats kept at 22° for 40 and 80 min. produced adrenal ascorbic acid depletions which were not significantly ($P<0.05$) less than those caused by the control samples. However, similar blood samples stored at the same temperature for 160 min. possessed only about one

TABLE I

STABILITY OF ENDOGENOUS CORTICOTROPHIN

Adrenal ascorbic acid depletions in hydrocortisone treated rats 1 hr. after intravenous injections (3 ml./100 g. body weight) of blood and plasma, before (controls) and after (tests) storage at various times and temperatures. Means±S.E. No. of rats in parentheses. The ascorbic acid level in the saline-injected controls was 505±11 mg./100 g. and in uninjected hydrocortisone treated rats 508±11 mg./100 g. adrenal tissue.

Material Assayed	Storage Temp. (°C.)	Storage Time (min.)	Mean Depletion (mg./100 g. Tissue±S.E.)	
			Control	Test
Blood from adrenalectomized rats	22	40	87± 6 (16)	81± 7 (17)
,, ,,	22	80	89± 7 (13)	91± 5 (16)
,, ,,	22	160	71± 8 (11)	29± 9 (9)
,, ,,	22	320	90±12 (6)	−16±20 (6)
,, ,,	4	160	70±11 (6)	52±11 (6)
,, ,,	4	320	90±12 (6)	20± 5 (6)
Blood from stressed adrenalectomized rats ..	22	40	139± 7 (6)	124± 9 (6)
,, ,,	22	80	139± 5 (24)	132± 8 (11)
,, ,,	22	160	139± 7 (6)	36±11 (6)
Plasma from stressed adrenalectomized rats	−15	24 hr.	98±11 (6)	121±13 (6)

TABLE II

COMPARISON OF PLASMA AND WHOLE BLOOD

Adrenal ascorbic acid depletions in hydrocortisone treated rats 1 hr. after intravenous injections (3 ml./100 g. body weight) of blood, plasma, saline washings, and suspensions of cell sediments from same samples. Means±S.E. No. of rats in parentheses.

Expt. No.	Material Assayed	Mean Depletion (mg./100 g. Tissue ±S.E.)
1	Whole blood from normal intact rats ..	−11± 7 (6)
	Plasma from same sample	−5±12 (6)
	Cell sediment from same sample resuspended in normal saline	16±14 (6)
2	Whole blood from stressed adrenalectomized rats	129±14 (6)
	Plasma from same sample	158± 9 (5)
	Saline washings of cell sediment ..	99± 8 (6)
	Washed sediment resuspended in normal saline	91± 9 (6)
3	Whole blood from stressed adrenalectomized rats	134±10 (6)
	Plasma from same sample	98±11 (6)
4	Whole blood from stressed adrenalectomized rats	109± 7 (6)
	Plasma from same sample	106±12 (6)
	Cell sediment from same sample resuspended in normal saline	57±10 (6)

half of the adrenal ascorbic acid depleting activity of the controls, and samples kept for 320 min. produced no fall in adrenal ascorbic acid. Storage of blood at 4° delayed slightly the rate of inactivation. Samples of blood kept at this temperature for 160 and 320 min. possessed about three-quarters and a quarter of the activity of the controls respectively. Similar results were obtained using blood from stressed adrenalectomized rats, but the adrenal ascorbic acid depleting activity of the control samples was greater. About 50% inactivation occurred at 22° after storage for 80 to 160 min. Plasma stored at −15° retained its entire activity for 24 hr.

Table II shows the results of experiments in which the adrenal ascorbic acid depletions produced by whole blood were compared with those due to plasma and washings and suspensions of the cell sediments from the same blood samples. Volumes of normal saline equal to that of the separated plasma were used to wash or suspend the sediments.

Blood, plasma, and cell suspensions from normal animals possessed no detectable activity. Whole blood from stressed adrenalectomized rats caused a pronounced fall in adrenal ascorbic acid concentration and the results of several experiments did not differ significantly ($P<0.05$). However, the results obtained using plasma were more variable. Considerable activity was left in the cell fraction, and saline washings and suspensions of the cell sediments produced marked adrenal ascorbic acid depletion. No explanation can be suggested for the anomalous result of experiment 2 (Table II), where the total activity in the plasma, washings, and suspensions of the cell sediments appeared to be greater than that in the whole blood. However, this is the only instance in our experience where the plasma corticotrophin level was markedly higher than that in blood.

DISCUSSION

There are relatively few stability studies on corticotrophin. Some investigators have examined the stability *in vitro* of exogenous corticotrophin added to blood. Reiss, Badrick, Halkerston, and Plaice (1951) reported the extreme instability of the hormone in such conditions, and Pincus (1951) observed the presence in blood of an enzyme capable of destroying corticotrophin. On the other hand, Richards and Sayers (1951), Pincus, Hechter, and Hopkins (1952) and Geschwind and Li (1952) found that incubation of corticotrophin with plasma did not result in any appreciable loss of activity. These discrepancies may possibly be explained on the basis that different preparations of the hormone, varying considerably in source and purity, were used in the investigations. Certainly the results yielded information of little value to workers concerned with the estimation of endogenously secreted corticotrophin in blood.

Direct estimates of circulating corticotrophin are now being used more frequently by laboratory workers investigating the physiology of the

pituitary-adrenal system. Furthermore, the development of simpler and more sensitive assay techniques makes it probable that routine methods for the estimation of blood corticotrophin will ultimately be available to clinical endocrinologists. It appeared, therefore, that the stability in blood of endogenously secreted corticotrophin should be systematically studied to obtain more information on the optimal conditions for the collection and storage of blood samples before the determination of their corticotrophin content.

Adrenalectomized and stressed adrenalectomized rats were used in the present study to provide the blood samples because their circulating levels of corticotrophin are sufficiently high to be estimated easily by the method described by Cox *et al.* (1958). The concentration of corticotrophin in the blood of rats three weeks after adrenalectomy is approximately 10 mU./100 ml. of blood (Cox *et al.*, 1958), and this high circulating level of the hormone is raised even further 2.5 min. after anaesthesia with ether (Hodges and Vernikos, 1959).

From a consideration of Table I it appears that there is no appreciable loss of adrenocorticotrophic activity when whole blood is stored at temperatures between 4° and 22° for periods of up to about 1 hr. or when plasma is kept at −15° for 24 hours. These results are in agreement with the findings of Paris (1954), who showed that plasma withdrawn from adrenalectomized rats possessed considerable adrenocorticotrophic activity 30 min. later. Montanari, Martinelli, Rossi, and Moruzzi (1951) reported that normal human plasma contained 160 mU. corticotrophin/100 ml. and that 49% of this activity was lost 4 hr., and 100% 12 hr., after storage at 18°. The validity of their results is placed in doubt by the fact that few other workers have been able even to detect corticotrophin in normal human plasma.

It was impracticable to study the stability of corticotrophin in whole blood stored at −15°, since changes, such as haemolysis, which occur make it too toxic to be tolerated by the assay animals. In our experience rat serum is also toxic when injected intravenously into rats in volumes greater than 0.5 ml./100 g. of body weight although Clayton, Hammant, and Armitage (1957) found that guinea-pig serum "led to anaphylactic reactions with death" only in doses greater than 2.0 ml./100 g.

Since only plasma can be stored in the conditions necessary for maximal stability of corticotrophin, it would appear desirable to perform corticotrophin assays on plasma rather than on whole blood. Furthermore, Barrett (1956) observed that the separation of plasma had the added advantage of concentrating corticotrophin added to whole blood, presumably since the added hormone was distributed only throughout the plasma. However, in our experience estimates of plasma corticotrophin are more variable than determinations of the hormone in whole blood. Our results also provide very little evidence that the concentration of corticotrophin is higher in the plasma fraction. It appears that much of the hormone remains associated with the cellular fraction, probably adsorbed on to the surface of the cells. This is consistent with the observation that considerable activity can be demonstrated in saline washings or suspensions of the cells.

These studies do not suggest how corticotrophin is inactivated by blood. The destruction of crude preparations of corticotrophin added to blood has been attributed to enzymes which they contain (Pincus *et al.*, 1952), and probably endogenous corticotrophin is inactivated similarly. The disappearance of corticotrophin from the circulation *in vivo*, however, occurs much more rapidly, and most estimates of the biological half-life of the hormone in blood vary between 1 and 5 min. (Greenspan, Li, and Evans, 1950; Gemzell, Van Dyke, Tobias, and Evans, 1951; Sydnor and Sayers, 1953; Sydnor, 1955).

Astwood (1955) considered that, since the increased content of corticotrophin in the blood following stress and adrenalectomy is thought to disappear from the blood at such a rapid rate, "rigorous precautions are essential to prevent this if assays are to be made." The results of the present series of experiments indicate that elaborate precautions are not necessary. Assays of circulating corticotrophin should be made on whole blood rather than on plasma. Excess heparin should be added to the blood samples to prevent clotting and the release of toxic substances, the samples should be kept cool, and the assays should be made within an hour of collection. If these very simple precautions are observed, estimates of circulating corticotrophin may be made with no reason for doubting their validity.

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THE EFFECTS OF SODIUM SALICYLATE ON THE OXYGEN CONSUMPTION OF RATS

BY

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The effects of sodium salicylate on oxygen consumption of intact rats and rats treated with tubocurarine have been studied. Intraperitoneal injections of sodium salicylate in untreated rats produced varying results which depended on both the dose and the concentration used. Intravenous injections in rats treated with curare gave rise to an increase in oxygen consumption which was proportional to the log dose over a range of 0.0625 mg./g. to 0.5 mg./g. body weight. It was concluded that sodium salicylate stimulated the oxygen consumption of rats when the concentration of the drug in the plasma was high.

In man, a rise in oxygen consumption after administration of salicylates has been demonstrated (Cochran, 1952). The interpretation of the effects of salicylates on the oxygen consumption of laboratory animals has, however, been complicated by the direct action of these drugs on respiratory rate and by changes in the degree of activity of the animals during the experiments. The present paper reports the effects of sodium salicylate on oxygen consumption by rats, untreated and treated with tubocurarine.

METHODS

Experiments with Untreated Rats. — Thirty-two male albino rats were divided into 8 groups of 4 each. Four groups received intraperitoneal injections of sodium salicylate solutions made up in two concentrations (10% and 2.5% w/v) which produced two blood concentrations, namely 0.5 mg./g. and 0.125 mg./g. body weight (see Table I). The remaining 4 groups received intraperitoneal injections of NaCl solutions of comparable molarity and volume. Oxygen consumption was recorded during three 30 min. periods (one before injection and two after), by a closed circuit method. The CO_2 evolved by the animal was absorbed in a soda-lime tower and the oxygen consumed was replaced from a calibrated spirometer. The change in volume of the spirometer, representing oxygen consumption, could be read to 2.2 ml. This resulted in a " reading error " of about 1% when oxygen consumption was calculated for a 30 min. period.

Experiments with Rats Treated with Tubocurarine. —Twenty-four male albino rats were divided into 6 groups of 4 each. Sodium salicylate was given intraperitoneally to 2 of the groups and intravenously to the remaining 4 groups. Under ether anaesthesia, the trachea was cannulated and 360 μg. tubocurarine chloride was injected into an external jugular vein; a further 180 μg. was given 1 hr. later. Ventilation was maintained by a pump with a stroke volume of about 5 ml. The expired air was directed by a valve system through a Pauling-Beckman oxygen analyser into a spirometer. The rate of the pump was regulated to maintain the oxygen tension of the expired air at about 130 mm. Hg. The oxygen consumption of the rats was calculated every 5 to 10 min. from the oxygen tension of the expired air and the ventilatory rate. By this method oxygen consumption could be determined to within 5%.

RESULTS

Untreated Rats.—The oxygen consumptions of the rats receiving NaCl solutions increased slightly during the first 30 min. after injection and decreased slightly during the second period, but these responses were not significant. The injection of 0.5 mg./g. body weight of sodium salicylate in 10% solution was followed by a 20% increase in oxygen consumption. When this dose was given in 2.5% solution there was no significant change in oxygen consumption. The administration of 0.125 mg./g. body weight sodium salicylate in 10% solution did not alter oxygen consumption significantly. However, when this dose was given in 2.5% solution a small but significant reduction (about 8%) in oxygen consumption occurred (Table I).

Rats Treated with Tubocurarine. — Intraperitoneal injections of 0.5 mg./g. body weight of sodium salicylate in 10% solution were followed by an increase in oxygen consumption of about 15%; when a quarter of this dose was given in

TABLE I

THE EFFECTS OF INTRAPERITONEAL INJECTIONS OF SODIUM SALICYLATE AND SODIUM CHLORIDE ON THE OXYGEN CONSUMPTION OF UNTREATED RATS

Oxygen consumption is mean ml./kg.$^{3/4}$±S.E.: for control period, total consumption; for test periods, mean difference (control period−test period). n=4 in all groups. All periods 30 min. Groups 1 and 5, 2 and 6, 3 and 7, 4 and 8 form comparable pairs with respect to molarity and volume of fluid injected.

Group	Mean Body Weight		Conc. %w/v	Dose (mg./g.)	O_2 Consumption		
					Control	After Injection	
	g.	kg.$^{3/4}$				1	2
Sodium salicylate							
1	200	0·299	10·0	0·50	561·8±31·1	+146·4±38·7	+77·4±23·8
2	233	0·335	10·0	0·125	525·5±26·4	+15·5±15·9	−32·4±18·9
3	219	0·320	2·5	0·50	648·3±23·0	+0·7±10·4	−17·0±17·7
4	228	0·330	2·5	0·125	608·8±28·0	−22·6± 9·9	−69·5±11·7
Sodium chloride							
5	225	0·327	3·6	0·18	611·0±38·7	+27·8±28·5	−25·1± 4·6
6	231	0·333	3·6	0·045	594·0±31·0	+22·7±12·2	−23·7± 7·5
7	221	0·322	0·9	0·18	611·0± 8·8	+21·1±19·4	−26·0±16·0
8	237	0·336	0·9	0·045	540·0±21·5	+24·9±18·5	−21·1±20·7

TABLE II

THE EFFECT OF INTRAPERITONEAL INJECTIONS OF SODIUM SALICYLATE ON THE OXYGEN CONSUMPTION OF RATS TREATED WITH TUBOCURARINE

Oxygen consumption calculated as in Table I. n=4 in both groups.

Group	Mean Body Weight		Conc. %w/v	Dose (mg./g.)	O_2 Consumption		
					Control	After Injection	
	g.	kg.$^{3/4}$				1	2
9	206	0·306	10·0	0·50	550·6±31·9	+80·8±25·9	+72·6±22·6
10	212	0·312	2·5	0·125	578·2±35·4	+31·1±43·3	+34·2±38·9

TABLE III

THE EFFECT OF INTRAVENOUS INJECTIONS OF SODIUM SALICYLATE ON THE OXYGEN CONSUMPTION OF RATS TREATED WITH TUBOCURARINE

Oxygen consumption calculated as in Table I. n=4 in all groups. (Two animals in Group 14 died during the final period.)

Group	Mean Body Weight		Dose (mg./g.)	O_2 Consumption		
				Control	After Injection	
	g.	kg.$^{3/4}$			1	2
11	223	0·324	0·0625	552·2±43·7	+25·4±26·6	−7·1±38·9
12	248	0·351	0·125	548·7±35·9	+50·8±37·1	+39·5±35·0
13	226	0·328	0·25	514·2±44·2	+150·5± 8·9	+116·4±21·3
14	213	0·313	0·50	538·9±47·6	+246·8±59·2	—

2.5% solution there was no significant change (Table II).

During the first 30 min. after intravenous injection of sodium salicylate, the change in oxygen consumption of the rats varied linearly with the log dose ($r=0.76$, $P<.001$). With the highest dose (0.5 mg./g. body weight), the increase in oxygen consumption was about 50% during the first 30 min. (Table III). Two of the rats, however, died during the second 30 min. after injection, presumably due to the toxic effect of the drug.

DISCUSSION

Interpretation of the results obtained from the untreated rats is subject to the limitations mentioned in the introduction. However, the results do show that, in rats, intraperitoneal injections of sodium salicylate can produce varying effects on oxygen consumption. The dose of sodium salicylate which reduced the oxygen consumption of the untreated rats produced no such response in those given tubocurarine (compare group 4, Table I, with group 10, Table II). Thus, it is possible that the reduction in the oxygen consumption of the untreated rats is an indirect effect due to some sedative action. Certainly the rats of group 4 were less active during the test period.

The results from rats treated with tubocurarine clearly indicate that sodium salicylate stimulated oxygen consumption. By extrapolation from Table III, the intravenous dose of sodium

salicylate necessary to produce a significant response is found to be about 0.18 mg./g. body weight. The concentration of the drug in the plasma falls gradually as it is distributed in the body fluid (Smith, Gleason, Stoll, and Ogorzalek, 1946). However, the initial concentration in the plasma is about 4.5 mg./ml. (plasma volume, 0.175 × weight as g.$^{.725}$: Wang and Hegsted, 1949). It is possible that the stimulating effect of sodium salicylate depends upon the attainment of a fairly high plasma concentration. The necessary concentration in the plasma may not be reached when dilute solutions are given intraperitoneally. This could explain the lack of response when 0.5 mg./g. body weight is given in 2.5% solution by this route.

The simplest explanation for the increase in oxygen consumption is to assume that adrenaline is released, possibly by a stimulating effect of the drug on the hypothalamus (George and Way, 1957). This hypothesis agrees with that put forward by Smith (1955) to explain the hyperglycaemia occurring in rats after the administration of salicylates. However, a direct action on tissue metabolism must also be considered as a possibility. Sodium salicylate is known to increase the oxygen uptake of rat cerebral cortex slices (Fishgold, Field, and Hall, 1951) and of liver slices in the presence of citrate (Alwall, 1939).

The view that the increased respiratory rate observed after large doses of salicylates is the result of direct central stimulation by the drug is well established (Smith, 1949). The present experiments do not make it necessary to alter this view. It may well be that the increases in oxygen consumption and in the rate of respiration represent separate effects of salicylates, as seems to be the case in dogs (Tenney and Miller, 1955).

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THE EFFECT OF TREATMENT WITH A LARGE DOSE OF ISONIAZID ON AN ESTABLISHED TUBERCULOUS INFECTION IN MICE

BY

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Mice were infected intracorneally with *Mycobacterium tuberculosis* var. *bovis* and the infection allowed to develop for a period of 2 weeks. At this stage the animals were divided into two main groups; one received no treatment, the other was treated with large doses of isoniazid (3.0 mg./mouse/day). The effect of treatment on the primary lesion, the viability of the bacilli, the systemic spread of infection, and the production of immunity were observed. Treatment was continued for 11 months, after which the animals were observed for another 8 months. Within a few days of starting isoniazid treatment the primary lesion stopped increasing in size, regressed slightly and stabilized at a size of about one-third of that of the controls. There was little evidence of any clearing of the bacilli from the lesions; they remained strongly acid-fast and morphologically normal for many months after infection, although by 13 months about half of the organisms in both groups had become very granular. The evidence suggests that in control and treated animals the bacilli in the cornea had usually all died within 8 months after infection. In two treated corneas, living virulent bacilli were demonstrated 15 months after inoculation. In the untreated animals, the disease spread systemically to involve the lungs, liver, and spleen, and by one year after inoculation the systemic tuberculous infection was very heavy, though not enough to kill the animals. The lesions in these animals contained many acid-fast bacilli. In the treated group, systemic spread of infection as judged by the development of small macroscopic lung foci was slight; acid-fast bacilli were found in only one animal. In the treated animals, practically no immunity could be detected 5 months after inoculation and had not reappeared 2 months after cessation of treatment; in the untreated animals immunity was present.

Prolonged chemotherapy in tuberculosis frequently fails to eradicate the infection. This has been demonstrated experimentally in animals and in clinical practice. Thus McCune and Tompsett (1956) infected mice intravenously and then treated them with various drugs and combinations of drugs for periods up to about 4 months, but were not able to eradicate the infection. Even with a combination of pyrazinamide and isoniazid which appeared to be most effective under those experimental conditions, there was no complete elimination of the bacilli (McCune, Tompsett, and McDermott, 1956).

In guinea-pigs it has been found that treatment of chronic lesions with streptomycin for 300 days can leave large cyst-like abscesses filled with purulent material in the lungs, liver, and spleen. These contain many viable tubercle bacilli which are still sensitive to streptomycin (Steenken, Wolinsky, Pratt, and Smith, 1952; Bojalil, Perez-Tamayo, and Bastarrachea, 1958).

Tubercle bacilli have also been recovered from healed human lesions after prolonged chemotherapy (Hobby, Auerbach, Lenert, Small, and Comer, 1954). Prolonged prophylactic chemotherapy with isoniazid in children with primary tuberculosis does not always prevent the disease spreading, though this occurs less frequently than in untreated children (Ferebee, Mount, and Anastasiades, 1957). The effect of prolonged chemotherapy of tuberculosis in man is discussed by Canetti (1955).

It seemed of interest to study the fate of a primary tuberculous lesion subjected to prolonged chemotherapy. Primary experimental

tuberculosis of the cornea in the mouse seemed to offer a satisfactory model lesion for this work because the cellular changes and bacterial content can be followed quantitatively by a variety of methods and the degree of immunity present in the animal can also be assessed.

After inoculation of the mouse cornea with *M. tuberculosis* there is rapid multiplication of the organisms and cellular invasion ; after 10 days the number of organisms is such as to elicit the formation of a macroscopically visible lesion. Subsequently, the lesion spreads to involve much of the cornea, although the total number of organisms does not increase much beyond that reached at about two weeks. After reaching a maximum the lesion decreases and reaches a size which remains steady for many months (Rees and Robson, 1950). In the present experiments mice were inoculated intracorneally and a macroscopic lesion allowed to develop. These mice were then treated with large doses of isoniazid over a long period and the development of the local lesion and the systemic spread were compared with untreated controls. Immunity was also investigated and an attempt was made to determine whether viable bacilli were present in the lesions in both groups of animals at various times after starting isoniazid treatment.

Methods

One hundred and ten albino mice of a mixed strain bred in our laboratories and about 3 months old at the start of the experiment were used. They were infected intracorneally by the method of Rees and Robson (1950) using a 1/100 dilution of a Dubos culture of *M. tuberculosis* var. *bovis* (3rd subculture ; 13th day), originally supplied by Dr. Levinthal, and since then maintained in our laboratory by the methods described by Acharya, Sullivan, and Robson (1958).

The eyes were examined at intervals for about 19 months macroscopically as described by Rees and Robson (1950) and microscopically by phase contrast microscopy and histological methods described by Robson and Sullivan (1957a). This included gross staining of the whole cornea with carbol fuchsin to reveal the localization and density of the bacillary mass, and counterstaining with methylene blue to show the extent and nature of the cellular content.

The disease was allowed to develop for 14 days, by which time lesions had appeared in all inoculated corneas, varying in size from 0.1 (just visible macroscopically) to 2.0 (occupying about two-thirds of the cornea) with an average of 0.6. These animals were divided into three main groups: (1) 13 mice with rather large corneal lesions: range 0.4 to 1.7, mean 0.9 ; (2) 62 mice with smaller lesions: range 0.1 to 1.0, mean 0.6 ; (3) 35 mice with lesions varying from 0.1 to 2.0, mean 0.6.

Groups (1) and (2) were treated with isoniazid (3.0 mg./mouse/day) mixed in M.R.C. diet 41. Group (3) was used as control and left untreated. Group (1), consisting of mice with large lesions, was specifically chosen to demonstrate a marked reduction in lesion size under intensive isoniazid therapy, should this occur. Most of the treated animals were given isoniazid continuously for 11 months. In two subgroups of animals treatment was discontinued 2½ months and 5 months respectively after infection, in the first instance to test for viability of organisms and in the second to test for the presence of immunity.

When used, cortisone was given as cortisone acetate, 0.5 mg./mouse/day subcutaneously.

Results

Effect of Isoniazid on the Corneal Lesion.—In Fig. 1 the average lesion size is plotted against time after infection. In the controls, the lesions appeared after the usual latent period with this size of inoculum (8 days) and gradually increased in size until after three weeks they occupied slightly more than half the cornea. The lesions then gradually regressed, leaving a chronic residual lesion occupying about a third of the cornea. In the treated animals, the lesions did not increase appreciably in size after starting treatment, and, after a latent period of about a week, they began to decrease to a size which was substantially maintained for the rest of the experiment. The decrease in size was rather greater in the group of 13 mice with a higher average lesion size when treatment was started (group (1)), so that both treated groups stabilized eventually at about the same level. In 5 mice which had maximum lesions ranging from 0.4 to 1.7 there was complete regression leaving the cornea clear macroscopically. When isoniazid was discontinued after various intervals from 2 months to 11 months, there was no evidence of breakdown of the lesions.

Microscopic Appearance of the Lesions.—The infected corneas of all groups of mice, treated and untreated, were examined at intervals after inoculation by phase contrast microscopy, gross staining of the eye (Robson and Sullivan, 1957a), and by normal histological techniques.

In the control group 50 days after inoculation, phase contrast microscopy showed a dense cell reaction consisting mainly of macrophages around and among the bacilli. There was also some vascularization. After gross staining, much of the macroscopic lesion was seen to consist of a heavy perifocal inflammation, mainly by macrophages.

FIG. 1.—The average size of corneal lesion in 3 groups of animals. The experiment was continued for 19 months, but few animals were left after 12 months. Treatment period: 0.5 to 11.5 months. X——X, Untreated mice; O——O, mice treated with isoniazid; ●----●, subgroup of treated mice with large lesions at the beginning of treatment.

In the treated groups, the lesion centre was qualitatively similar to that of the control group, with many macrophages containing bacilli and also vascularization. The cornea surrounding the lesion was relatively acellular, and after gross staining very little perifocal inflammation was seen.

The same appearance was found in eyes from the treated group examined five and six months after infection. After gross staining, bacilli and also acid-fast debris corresponding exactly in their distribution to the shape of the macroscopic lesion was seen and there was very little perifocal inflammation. Most of the bacilli were normally stained, but others consisted of a number of dots; occasionally acid-fast globules were all that remained of a bacillus. Up to this time there was no evidence of any decrease in the number of bacilli. By 7 months the presence of granular bacilli and acid-fast globules became more marked, though many organisms of normal shape were still present in most of the eyes.

Eight months after infection, an animal from the treated groups was killed and its cornea examined. When treatment was started, this animal had had a corneal lesion occupying one-fifth of the eye. After treatment for three weeks, this had regressed completely and the eye had been macroscopically clear for the following seven months. However, there was microscopic vascularization and a small lesion 300 μ in diameter consisting mainly of macrophages (some with intracellular bacilli) and a few granulocytes. When stained many morphologically normal and a few very granular bacilli were seen. There were relatively few cells and these were confined to the site of the bacilli.

By 13 months after inoculation, there was no essential change in the histological appearance. Bacilli were still intracellular in macrophages and by this time were roughly divided so that half were morphologically normal and half granular. There was, however, still no evidence of any decrease in the total number of bacilli present in the eyes. At this time there were more invading cells in the corneas of the control than of the treated groups, but the morphology of the bacilli was identical in both. During the whole of this time there was no detectable difference in the acid-fastness of the bacilli in treated and untreated mice.

Fifteen months after infection, corneas from the control and treated groups showed no qualitative differences. In both there was corneal fibrosis with vascularization and large foci of inflammatory cells, almost exclusively macrophages, but also some lymphocytes and very occasional polymorphs. Bacilli, many morphologically degenerate, were also seen in the cell foci.

Effect on Viability of Organisms.—When mice inoculated in the cornea were treated with 0.3 mg. of isoniazid/mouse/day for 36 days and treatment was then stopped, lesions appeared after a latent period of about 11 days. When the daily dose of isoniazid was 3.0 mg. the latent period was considerably longer (Robson and Didcock, 1956). Furthermore, living and fully virulent tubercle bacilli could be recovered from such a cornea after 50 days of treatment. In other experiments, cessation of treatment with the higher dose of isoniazid after 28 days was not followed by breakdown during a subsequent observation period of three months (Goulding, 1956). The present experiment allowed the viability of

bacilli to be investigated after longer periods of treatment.

In one subgroup of six mice, treatment was stopped after 62 days of treatment, and observations continued for about four months. During this period there was no change in the size of the macroscopic lesions. To determine whether any live organisms were present in these corneas, three of the animals were treated with cortisone during the last five weeks of the period, since it has been shown that cortisone can reactivate a chronic corneal lesion in the mouse (Robson and Sullivan, 1957b). No such reactivation was seen in the present experiment; in one animal there was an appreciable decrease in the size of the lesion. There was no evidence of multiplication of bacilli or of cord formation.

Eight months after the beginning of the experiment another attempt was made to determine whether viable organisms were present. This was done in two subgroups of animals, one treated with isoniazid and the other controls. The animals were killed and the corneas dissected, washed four times with sterile saline and put into Dubos medium containing penicillin (50 units/ml.). In corresponding eyes examined microscopically at this time many bacilli were present within macrophages; the majority of the bacilli were morphologically normal although there were many granular bacilli present. None of the six treated animals killed at this time showed any evidence of systemic infection, either macroscopically or microscopically; of the four control animals, two had heavily infected lungs containing many acid-fast bacilli, the majority of which were granular or polar.

After 23 days, one of the corneas removed from the treated subgroup was taken from the Dubos medium and examined by phase contrast microscopy and then stained with carbol fuchsin. Practically all the epithelium had fallen from the cornea, but a few invading cells in the stroma were still intact and some intracellular bacilli were easily seen. After staining, many extracellular and intracellular bacilli were visible and of normal appearance, but there was no sign of multiplication or cord formation. At the same time all the other eyes were transferred into fresh Dubos medium without penicillin. The Dubos medium in which the eyes had been incubated for three weeks was transferred to centrifuge tubes and spun at 3,000 rev./min. for 20 min. The deposit was stained by the Ziehl-Neelsen method, but no bacilli were found in any of the tubes. After incubation for a further two weeks, all the eyes were removed from the Dubos medium and examined by phase contrast microscopy and stained. By phase contrast, many bacilli were seen to be present in all the eyes of both subgroups. These were easily seen, but cell structure was difficult to define by this time, although some morphologically intact macrophages were still visible. After staining, many bacilli were found in both subgroups of eyes with rather more in the controls than in the treated group. The controls all had larger lesions than the treated group and this difference in numbers of bacilli would be expected. There was no evidence of cord formation or of multiplication of the bacilli. At the same time as the viability was being tested, two control animals and two treated animals were killed and the corneas removed and implanted subcutaneously in the groin of four guinea-pigs. Ten months later these guinea-pigs were killed and examined. All animals were completely normal with no evidence of tuberculosis.

Treatment with isoniazid was stopped in all animals after 11 months. Two months later another viability test was carried out. Four animals from groups (2) and (3) were taken, the corneas removed and digested in a collagenase: hyalase solution for 18 hr. at 37° (Robson and Smith, 1959) to release the bacilli. The digest was then transferred to Kirchner medium and incubated at 37°. After culture for seven weeks there was no evidence of growth in any of the tubes. One tube from each group was subcultured into Dubos medium; after three weeks there was still no sign of growth.

One month later (14 months after the beginning of the experiment) another 4 animals were taken from the same 2 groups and the corneas removed and digested in collagenase: hyalase as before. In addition a piece of lung and spleen from each animal was similarly digested. A drop of each of these digests was placed on to Löwenstein-Jensen and Dorset egg-slopes, and into Dubos medium. Four guinea-pigs were also given injections into the lung and spleen, 2 with digests of a cornea from the control and treated groups, and 2 with digests of lung and spleen from the treated group only. Six months later these guinea-pigs were killed and showed no evidence of tuberculosis. In the cultures there was no growth from the digests from the treated animals, but tuberculous colonies grew on 2 of the Löwenstein-Jensen slopes from the lung and spleen digests from control animals.

One month later (15 months after the beginning of the experiment) another 2 mice from the same groups were killed, and their corneas were

removed and implanted subcutaneously in the groin of 4 guinea-pigs. The 2 guinea-pigs implanted with the treated corneas died from generalized tuberculosis, one after 3 months and the other after 4 months. Organisms from the liver of the animal which died after 3 months were cultured on Löwenstein-Jensen slopes and thereafter kindly tested by Professor Knox for sensitivity to isoniazid. They were sensitive to 0.08 μg./ml. of isoniazid as was the original parent strain used for inoculation. The guinea-pigs implanted with the untreated corneas survived and appeared normal until they were killed for examination 5 to 12 months later: there was no evidence of tuberculosis.

Nineteen months after infection (8 months after cessation of treatment) two treated animals survived which had shown no breakdown of corneal infection. Their corneas, as well as that of a control mouse, were implanted into guinea-pigs. One of the treated eyes which had no macroscopic lesion showed a small lesion under phase contrast. This consisted of an aggregate of cells containing a number of clearly seen bacilli. These guinea-pigs were killed after 4.5 months and there was no evidence of tuberculosis in any of them.

Effect of Treatment on the Systemic Spread of the Disease.—Previously it has been shown that very little systemic spread of infection occurs in mice after intracorneal inoculation with virulent bovine tubercle bacilli. This has encouraged the use of the intracorneal method for testing anti-tuberculous drugs, since the lesion in the eye is a closed one and there is little risk to man in handling the animals. This lack of spread is certainly true if the animals are kept for only the few weeks necessary to perform a screening test, but we have found that if animals are kept for many months systemic spread occurs and extensive lesions are produced, mainly in the lungs. We were therefore interested in studying, as part of this investigation, the effect of isoniazid treatment on the systemic spread from a corneal lesion.

Eight months after infection, 6 treated animals and 4 controls were killed and examined. In the treated group, 5 animals had many small macroscopic foci of infection on the lung surface; apart from the cornea, they had no other macroscopic evidence of disease. Smears of the lung, liver, and spleen, stained by the Ziehl-Neelsen method, revealed no bacilli despite intensive search. All 4 of the control group had lung lesions of which 2 were considerably more extensive than in any of the treated animals. There was no other macroscopic evidence of disease. Many acid-fast bacilli were found in the lung smears from the 2 animals with extensive lung lesions, but not in the other animals. No acid-fast bacilli were found in smears of liver and spleen.

By 13 months after infection, there was evidence that the anterior chamber had become affected in some of the animals (5 of 15 controls and 3 of 21 treated animals). Five control and 5 isoniazid treated animals were examined after killing at that time (8 of these were also used for the viability tests). In the control group 4 out of the 5 had a heavy lung infection with many coalescing foci over the whole surface of the lung. The spleen of one had macroscopic lesions, but the livers and spleens of the others were macroscopically normal. The fifth animal also had a heavy lung infection with many discrete foci over the whole surface of the lung. The liver and spleen of this animal were normal. Many bacilli were found in smears from the lung of all 5 animals, from the spleen in 2, and from the liver in 2. In the treated group 2 of the 5 had very few (3 to 6) small lesions in the lungs, but the livers and spleen were normal. In the other 3 there was no macroscopic evidence of infection. Smears were made of the lungs, liver, and spleen and stained, but no acid-fast bacilli were found.

Fourteen months after infection one animal from each group was killed and various organs removed for histological examination. In the control animals the lung showed alveoli packed with large foamy macrophages and a few polymorphs; cholesterol clefts were also seen. In the lung substance there were multiple foci of chronic inflammatory cells, mainly macrophages and lymphocytes, but few polymorphs. There was no evidence of necrosis. In Ziehl-Neelsen stained sections there were many bacilli, particularly in the foamy macrophages in the alveoli. The appearance of the lung was that of a typical segmental tuberculous broncho-pneumonia possibly suggestive of a blood-borne infection. In the liver there was much infiltration of the portal tracts by macrophages and a few plasma cells. No tubercle bacilli were seen. Tiny foci were also found in the kidney cortex and the heart; the spleen was normal.

In the treated animal there was little evidence of disease in the lungs; there was some emphysema, but there were no large cell masses of infiltration, no foamy macrophages, and no

cholesterol clefts. No bacilli were found in the sections, and there was little to suggest tuberculous disease. In the liver there was infiltration around the portal tracts, though less than in the control. This consisted mainly of macrophages with a few plasma cells, polymorphs, and eosinophils. Again there were tiny foci in the kidney and heart consisting of macrophages and a few plasma cells. The spleen was normal. No bacilli were found in the sections from this animal. At the same time that animals were taken for the histological examination, 4 from each group were killed and parts of various organs were cultured for viable bacilli. In smears of the lungs, liver, and spleen stained with carbol fuchsin, organisms were found only in the lungs of 3 control animals and no acid-fast bacilli were found in any of the smears from the treated group. There was definite evidence of spread of viable bacilli to the lungs of some control animals, but not in the treated group. Two weeks after these animals were killed 2 treated animals died from an intercurrent infection and acid-fast bacilli were found in a smear of the lungs of one of them. This is the only time that we have found acid-fast bacilli in the organs (apart from the cornea) of a treated animal.

The Effect of Treatment with Isoniazid on Immunity.—It has previously been shown by Acharya *et al.* (1958) that tuberculous infection of one cornea in the mouse rapidly leads to the development of immunity which will prevent the development of a lesion if the animal is reinfected in the other eye. These authors showed that this immunity persists for at least 13 months after primary infection. By one week after infection the immunity was sufficient to prevent lesions developing in animals reinfected in the other eye with a 1:1,000 dilution of a Dubos culture of *M. tuberculosis* (about 500 bacilli). In the present experiment, treatment with isoniazid was started 2 weeks after infection, so that by then sufficient immunity would be present to inhibit completely the formation of lesions in a group challenged with the size of inoculum used. Five months after infection (4.5 months after the commencement of treatment) 2 groups of 6 mice, from groups (2) and (3), were reinfected in the other eye with a 1:1,000 dilution of *M. tuberculosis* var. *bovis*. In the group of mice which had received no isoniazid, no lesions had developed in any of the challenged eyes one month after challenge and after 9 weeks a small lesion had developed in only one animal. Of the treated group in which isoniazid was discontinued before challenge, 4 animals had developed lesions by 3 weeks after reinfection, and by 9 weeks another had developed a lesion. The eye of the remaining animal remained clear up to 9 months later. Thus, after treatment with isoniazid lasting for 4.5 months, there was a marked difference in immunity between the control and treated groups.

Thirteen months after infection (2 months after stopping treatment) the presence of immunity was again investigated in the treated animals, the second eye being challenged with a 1:1,000 inoculum. Three of the 6 animals had developed lesions in the challenged eye by the 14th day; one month after reinfection, all 6 animals had developed lesions. Thus in this group there had been practically complete suppression of the immunity by the treatment. Control animals, challenged in the same way, were unfortunately killed prematurely.

In conclusion, therefore, it may be said that after 5 months of treatment, while control animals still showed almost complete immunity, the immunity of the treated group had almost all gone, and after 13 months, while the controls were still probably immune (Acharya *et al.*, 1958), in the treated group all the immunity had disappeared.

Discussion

Intensive therapy with isoniazid produced marked effects on a progressive, established tuberculous lesion in the mouse. Treatment started 14 days after infection prevented further increase in size of the lesions; after about a week the lesions decreased somewhat in size and then stabilized. Prolonged treatment did not produce any further decrease in lesion size. In untreated mice the lesions increased in size during the first month after infection, then regressed slightly and stabilized at a size more than twice that of treated animals. This final difference in size can be explained by two effects of the treatment. Firstly, the further increase in the main core of the lesion (the mass of cells containing bacilli) which occurred in the control animals after the first fortnight was inhibited in the treated group; secondly, perifocal inflammation disappeared completely under the impact of therapy, but was maintained indefinitely in the controls. On the other hand, it is clear that prolonged treatment had no significant effect on the established focal lesion, all the bacilli remaining intracellular within a core of cells.

Within a few months of inoculation few viable bacilli remained in the corneal lesions of either

the treated or the control groups. Treatment had no effect on the acid-fastness, morphological appearance, degeneration or removal of the bacilli from the lesion, although it arrested their multiplication. The puzzling fact remains, however, that in the control eyes the perifocal inflammation persisted, whereas it cleared up quickly under treatment. This raises the highly speculative suspicion that the untreated bacilli, though non-viable when tested by methods involving culture or inoculation into guinea-pigs, nevertheless still retain some biochemical activity which maintains the perifocal inflammation and that this activity is suppressed by isoniazid. It is possible, of course, that the effect of isoniazid on the perifocal inflammation is not due to an action of the drug on the tubercle bacilli only, but also to an anti-inflammatory effect, since there is evidence that isoniazid can diminish inflammation (Spain, 1953; Theobald, 1955). It must, moreover, be emphasized that two corneas removed from animals three months after stopping isoniazid treatment (14 months after infection) produced tuberculosis when inoculated into guinea-pigs. This finding supports the results of other investigators (see Canetti, 1955) that tubercle bacilli can survive very prolonged chemotherapeutic action.

The observed inhibition of systemic spread must represent an important aspect of the action of isoniazid. There is little reason to suppose that isoniazid would inhibit the migration of bacilli from the primary focus, but it would effectively suppress any multiplication if these bacilli were deposited at other sites. It is probable, therefore, that bacilli in small numbers did reach the lungs and other organs of the treated animals, but failed to multiply. Further, they must have been rendered incapable of multiplication within the host because there was no evidence of breakdown in the 8 months of observation after the end of treatment.

The gradual loss of immunity in the treated animals must presumably be due to removal of the antigenic stimulus by the treatment. The total effect, however, is beneficial, since in spite of the disappearance of immunity there was no recrudescence of the disease. These experimental results in mice are in agreement with the clinical findings of Ferebee *et al.* (1957), who found that prophylactic isoniazid therapy in children with asymptomatic primary tuberculosis decreased the incidence of serious extrapulmonary tuberculous complications. Here too the therapy with isoniazid must have prevented the spread of infection from the primary focus.

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SUPERSENSITIVITY DUE TO PROLONGED ADMINISTRATION OF GANGLION-BLOCKING COMPOUNDS

BY

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Repeated subcutaneous injections of ganglionic blocking agents in cats were found to cause a supersensitivity towards adrenaline in the submaxillary gland and the nictitating membrane. The supersensitivity resembled that produced by preganglionic denervation, namely section of the chorda tympani in the case of the gland and of the cervical sympathetic trunk in the case of the membrane. The blocking compounds used were chlorisondamine chloride and hexamethonium. With the former, the sensitization could be obtained in the normally innervated structures. When hexamethonium was used, however, the effect on the gland could be produced only if the synapse had been made particularly susceptible to the blocking action of this agent; this was achieved by cross-suture experiments.

By long-continued treatment of an animal with atropine or a drug with atropine-like actions, it is possible to create a supersensitivity to chemical stimuli in the submaxillary gland (Emmelin and Muren, 1950, 1951, 1952; Emmelin, Jacobsohn and Muren, 1951; Emmelin and Henriksson, 1953; Emmelin and Strömblad, 1957), in the parotid gland (Strömblad, 1956a and b; Nordenfelt and Ohlin, 1957) and in the smooth muscle of the stomach (Muren, 1957). The supersensitivity observed in these cases resembles that which appears after section of the efferent nerve of the structure. Obviously, it would be of importance to know whether, as a general rule, a prolonged administration of blocking agents gives rise to a supersensitivity similar to that which follows denervation. To test this, some experiments were carried out in which the effect of prolonged treatment with a ganglionic blocking agent, hexamethonium, on the sensitivity of the submaxillary gland was investigated (Emmelin, unpublished observations). No sensitization took place. Similarly, Konzett and Rothlin (1953) found no supersensitivity of the nictitating membrane of the cat towards adrenaline or noradrenaline after the administration of hexamethonium for some time.

It seemed possible that these failures could be due to insufficient blocking of ganglionic transmission. In the present investigation, an attempt was made to repeat these experiments under more favourable conditions.

Two types of experiments were carried out. It has been observed that it is possible, under special conditions, to block very effectively and for a considerable period of time the transmission of nerve impulses to the postganglionic parasympathetic neurone which supplies the submaxillary gland with secretory fibres (Emmelin, Muren, and Strömblad, 1957). In cross-suture experiments, the preganglionic fibres of the chorda tympani were exchanged for somatomotor fibres from the hypoglossal nerve. The supersensitivity created by the section of the chorda decreased when the hypoglossal fibres had replaced the chorda fibres functionally. Hexamethonium in very small doses was now found to block the transmission of impulses from the hypoglossal nerve to the postganglionic neurone for a remarkably long period of time. This finding was made use of in the present investigation.

When these experiments had been completed, more suitable ganglionic blocking compounds than hexamethonium became available. Chlorisondamine chloride (ethylene-1-[4, 5, 6, 7-tetrachloro - 2 - methyl*iso*indolinium] - 2 - trimethylammonium dichloride, Ecolid [Ciba]) has a long duration of action; in addition, the striking tolerance which develops to hexamethonium is not seen with chlorisondamine. This

drug was therefore used in a series of experiments in which the sensitivity to adrenaline of the submaxillary gland and the nictitating membrane was examined.

Methods

In cats, which were anaesthetized with ether and hexobarbitone, the central end of the right hypoglossal nerve was sewn to the distal end of the chorda-lingual nerve. The sensitivity of the two submaxillary glands towards adrenaline was estimated at intervals in hexobarbitone anaesthesia. The salivary ducts were cannulated in the mouth. Hexobarbitone and adrenaline were administered intracardially. When the supersensitivity subsequent to the trans-section of the chorda-lingual nerve had declined (as a result of re-innervation from the hypoglossal nerve by way of the parasympathetic pathway), treatment with hexamethonium was instituted. The drug was injected subcutaneously twice daily over a period of two to three or more weeks. The initial dose, usually 3 mg./kg., was increased successively until a dose of 20 mg./kg. was reached; this dose was then maintained for some days. In some cats, the treatment started with 10 mg./kg. and the dose was raised to 20 mg./kg. The sensitivity was estimated 5 to 19 hr. after an injection of hexamethonium. In some cats, a later treatment with an atropine-like drug was given for a comparison, 1 mg./kg. of the compound Hö 9980 Hoechst (piperidino-ethyl-diphenyl-azetamid) being injected subcutaneously once a day as described by Emmelin and Henriksson (1953).

In the second type of experiments the sensitivity of the submaxillary glands was tested repeatedly as described above, but in addition the responses of the nictitating membrane to adrenaline were recorded under standardized conditions. A fine clip was placed at the edge of the membrane and connected to a frontal writing lever by a thread and a pulley. Chlorisondamine was injected subcutaneously twice a day in a dose of 2 mg./kg. over a period of some weeks. In some experiments, the dose was successively raised up to 5 mg./kg. The treatment was then discontinued, and when a pre-treatment degree of sensitivity had again been reached the effect of section of the chorda tympani and the cervical sympathetic trunk and of removal of the superior cervical ganglion was studied.

Results

Cross-suture Experiments

In a series of cats prepared with cross-suture, 7 survived for a sufficiently long period to allow the treatment with hexamethonium. Fig. 1 shows the outcome of one of the experiments. About three months after the hypoglossal nerve had been connected to the chorda-lingual nerve, the

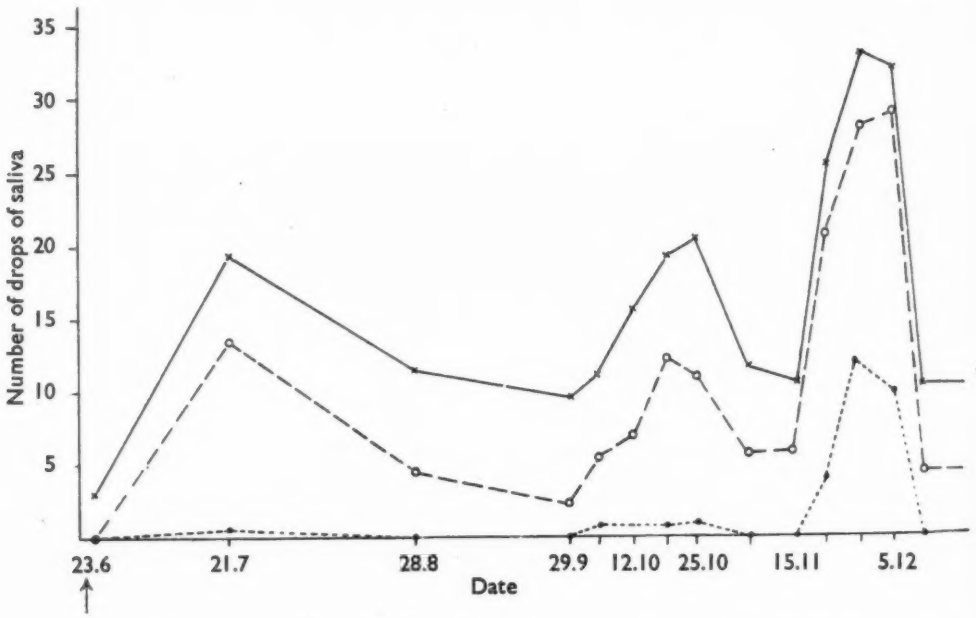

Fig. 1.—The sensitivity of the right submaxillary gland of a cat towards three different standard doses of adrenaline, in the upper curve 10, in the middle curve 5, and in the lower curve 2 μg./kg., tested on 14 occasions during a period of about six months in 1956. The right hypoglossal nerve was cut and its central end sewn to the peripheral end of the cut right chorda-lingual nerve at an operation on 23.6.56 (at arrow). Hexamethonium chloride was injected subcutaneously twice daily between 29.9 and 25.10. The dose given each time was 10 mg./kg. during the first, 20 mg./kg. during the second half of this period. Between 15.11 and 5.12, Hö 9980, 1 mg./kg., was injected once a day. The abscissa gives dates, 21.7 indicating 21st July, 1956.

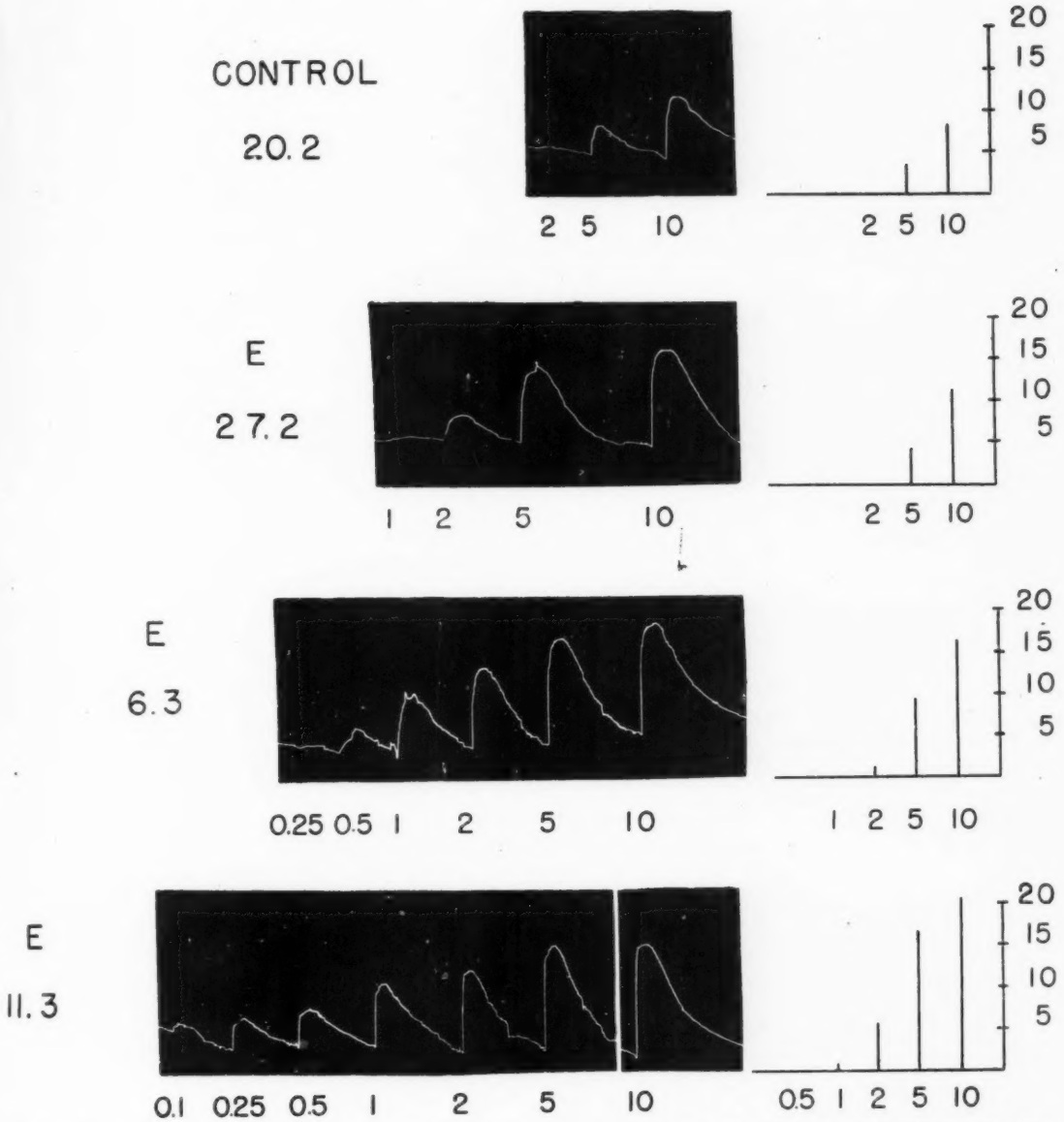

FIG. 2.—The sensitivity of the right nictitating membrane (smoked paper records on left) and of the right submaxillary gland (diagrams on the right) of a cat. The numerals below the tracings and the diagrams represent the doses of adrenaline in μg./kg., used to estimate the sensitivity. The ordinates of the diagrams give the number of drops of saliva secreted. After the control experiment on 20.2.58 (20.2), chlorisondamine (E) treatment was started. Two subcutaneous injections were given daily, the dose being raised successively from 2 to 5 mg./kg.

supersensitivity had been considerably reduced, although not quite abolished. During treatment with hexamethonium the sensitivity again rose and reached a maximum within three weeks of treatment, namely in about the period required for a maximum to be attained after section of the chorda. When the treatment was discontinued, the sensitivity fell and reached its previous level in a few days' time. Treatment with the anti-parasympathetic agent caused a more pronounced rise in sensitivity than that seen during administration of hexamethonium.

It is obvious from Fig. 1 that the supersensitivity caused by the treatment with hexamethonium was very similar to that following parasympathetic decentralization. This was true of all the various doses of adrenaline tested. Similarly the threshold, which had been lowered after the

operation but had risen again in the course of the re-innervation, was lowered when hexamethonium was given ; the decrease was the same in both instances. During treatment with Hö 9980 there was again a lowering of the threshold which was more pronounced than on the two previous occasions.

In the remaining six cats which had hypoglossal-chorda-lingual anastomosis, hexamethonium was also found to cause a rise in sensitivity. In one of these cats, it reached the degree observed earlier after section of the chorda, just as in the experiment shown in Fig. 1. In the other five cats, the rise was not quite as large as that caused by the operation.

In the seven cats of this series there was no increase in the sensitivity of the contralateral, normally innervated gland during the treatment with hexamethonium.

Treatment with Chlorisondamine

During the period when chlorisondamine was injected, the mucous membrane of the mouth was found to be very dry and no salivation seemed to occur when ether was given as the preliminary to the hexobarbitone anaesthesia.

Chlorisondamine, contrary to hexamethonium, was found to sensitize normally innervated structures. Fig. 2 shows responses to a series of doses of adrenaline of the right submaxillary gland (diagram to the right of Fig. 2) and the right nictitating membrane (records on the left of Fig. 2) on four different occasions, with weekly intervals. Treatment with chlorisondamine increased the sensitivity of both structures, the effect being maximal after about three weeks. The threshold doses were lowered, and the effects of moderate doses increased. When the treatment was discontinued the sensitivity fell again. In the submaxillary gland, the pre-treatment level was reached within a week ; in the nictitating membrane some supersensitivity persisted at that time, but after about two weeks the original level had been resumed. When the preganglionic nerves (the chorda and the cervical sympathetic) were then cut, it was found that the treatment with chlorisondamine had invariably sensitized the membrane to the same degree as had section of the cervical sympathetic, whereas in the submaxillary gland the chorda supersensitivity level was not quite reached. It seemed somewhat easier to sensitize the membrane than the gland with chlorisondamine. In one cat, two periods of treatment were given. In the first, the highest dose given was 2 mg./kg. twice a day. The gland was only slightly sensitized, but the membrane was sensitized to the level reached later after preganglionic denervation. In a second period of treatment the dose was raised to 5 mg./kg. The sensitivity of the membrane did not increase more than in the first period ; that this was not the highest level attainable was shown at the end of the experiment, when removal of the superior cervical ganglion caused a supersensitivity much higher than that produced by treatment with chlorisondamine or section of the preganglionic fibres. During the treatment with 5 mg./kg., the gland was much more sensitized than when the smaller dose was given, but chorda section was then found to cause a somewhat more pronounced supersensitivity. It seems reasonable to assume that the degree of sensitivity following section of the chorda would have been reached with a larger dose of chlorisondamine, but no cats survived such treatment.

Injection of chlorisondamine in an acute experiment did not affect the sensitivity of the membrane or the gland.

Discussion

The experiments showed that treatment with ganglion-blocking agents can produce a supersensitivity in gland cells and smooth muscle. The threshold was lowered by the treatment, and the response to a moderate dose of the stimulating agent was increased. In these respects, and in the time course of development, the supersensitivity resembled that brought about by denervation.

The level of sensitivity reached in these experiments was, at most, that attained after preganglionic denervation (decentralization). This is obviously the degree of supersensitivity which can be produced by depriving the effector cells of impulses from the central nervous system. The blocking action of Hö 9980 which acts more peripherally was followed by a much more pronounced supersensitivity in the submaxillary gland. This latter treatment has been assumed to be equivalent to postganglionic denervation (Emmelin and Strömblad, 1957). Using blocking agents acting in different points of the pathway, it is thus possible to imitate the conditions of surgical decentralization and denervation. Cannon's law of denervation, according to which postganglionic section causes a more pronounced supersensitivity than preganglionic section, seems to be applicable even when the "denervation" is brought about by pharmacological means.

Hexamethonium was kindly supplied by A. B. Leo, Hälsingborg, and Hö 9980 by A. B. Webass, Göteborg. Technical assistance was given by Miss Ursula Delfs.

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EFFECTS OF CROSS-SUTURE AND OF INJECTED ACETYLCHOLINE ON THE SUPERSENSITIVITY OF DENERVATED STRIATED MUSCLE

BY

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The sensitivity of the tongue muscles to acetylcholine was examined in repeated experiments on cats anaesthetized with hexobarbitone. The supersensitivity which developed after section of the hypoglossal nerve was found to decrease when the muscles, after cross-suture, became re-innervated from the vagus or the chorda-lingual nerve. The supersensitivity caused by denervation could also be reduced by injecting acetylcholine repeatedly during six hours in acute experiments.

Re-innervation of the submaxillary gland is accompanied by a decline in the supersensitivity to chemical stimuli which the gland cells develop after section of the chorda tympani. This was found to be the case even when the regenerating nerve was not the chorda but the hypoglossal nerve, indicating that the ordinary low degree of sensitivity is not maintained through some action specific to the normal secretory nerve (Emmelin, Muren, and Strömblad, 1957). However, the fibres cut were preganglionic, and experiments with hexamethonium showed that the hypoglossal fibres, connected to the chorda in a cross-suture, had only regenerated to the synapse. The secretory cells were therefore still innervated by the normal postganglionic fibres. In the present experiments, this complication caused by the presence of an intercalated synapse was avoided by working on striated muscle and its motor nerve. The muscles of the tongue re-innervated from the vagus or chorda-lingual nerves were studied.

The supersensitivity of a parasympathetically decentralized submaxillary gland can, likewise, be abolished by injecting repeatedly over several days a secretory agent such as pilocarpine or carbachol (Emmelin and Muren, 1950, 1951). Corresponding experiments were now carried out for a shorter period on the denervated muscles of the tongue, acetylcholine being used as a stimulating agent.

METHODS

The sensitivity of the tongue muscles of the cat was studied repeatedly in the same animal under standard conditions. The cats were anaesthetized first with ether, then with hexobarbitone given intracardially. With the cat on its back, the head fixed, and the mouth widely opened, a clip was placed at the tip of the tongue and connected by a thread and pulley to a lever writing on a smoked paper. When no hexobarbitone was needed, saline was injected intracardially to prevent clotting in the injection needle, and a series of doses of acetylcholine was administered by the same route. At the beginning of the experiment, 2 mg. of atropine sulphate was given intracardially to abolish the muscarinic effects of acetylcholine.

For the cross-suture experiments the hypoglossal nerve and the cervical vagus or chorda-lingual nerves were exposed on the right side using hexobarbitone anaesthesia. The nerves were cut and the central stump of one of the two latter nerves was sewn to the peripheral stump of the hypoglossal nerve. The sensitivity of the tongue muscles was then estimated at intervals and finally an acute experiment was carried out using chloralose anaesthesia. The result of the cross-suture was now examined by stimulating the nerves electrically.

The effect of often repeated injections of acetylcholine on the level of sensitivity was studied in acute experiments. The hypoglossal nerve was cut and, when a pronounced supersensitivity had developed, the cat was anaesthetized with chloralose. Atropine (2 mg.) was given intravenously, and during about 6 hr. large doses of acetylcholine (50 μg./kg.) were injected intravenously every 5 min. The sensitivity was then estimated at hourly intervals, using a convenient standard dose of acetylcholine, which was given intracardially.

RESULTS

The Development of the Supersensitivity

When the hypoglossal nerve had been cut on one side, a supersensitivity could first be observed

2 or 3 days after the operation. The sensitivity then increased quickly and reached a maximum on the sixth or seventh day. It then remained high, provided that no regeneration took place. The threshold dose of acetylcholine injected into the left ventricle was found to be lowered markedly, from 50 to 100 μg./kg. in the normal innervated, to 0.2 to 0.5 μg./kg. in the denervated structure. The effects of moderate doses of acetylcholine increased markedly during the first week following the denervation. The quick responses to acetylcholine were particularly affected. The slow component, caused in our experiments by doses of acetylcholine of the order of 10 to 20 μg./kg. and above seemed to appear on the fourth day, as described by Brown (1937), and increased in size during the following days; sometimes the slow response was superimposed upon the quick one, sometimes it appeared when the muscles were relaxing after the quick contraction, and sometimes not until the muscles had fully relaxed. The development of the supersensitivity during the first six days is illustrated in Fig. 1.

Cross-suture Experiments

Twelve cats with a vagus-hypoglossal suture and two with a chorda-lingual-hypoglossal suture survived for a month or more after the operation. In all these cats a pronounced supersensitivity developed as a consequence of the denervation, and later in all of them the sensitivity started to decline. In one cat the fall in sensitivity was first observed 38 days after the operation, but usually it was not found until after 6 to 10 weeks. The sensitivity then decreased progressively in the course of the following weeks to near the original level.

In the acute experiment, stimulation of the right vagus nerve in the cats with the vagus-hypoglossal suture was observed to cause a contraction of the tongue, often as pronounced as that caused by excitation of the left hypoglossal nerve. Stimulation of the left vagus gave no contraction. However, in most experiments it was possible to obtain a small contraction by stimulating the right hypoglossal nerve, indicating some degree of re-innervation from the normal motor nerve. When this was first observed the precaution was taken in the later experiments to free a long stretch of the hypoglossal nerve by careful dissection, to cut it as far distally as possible and to displace the central stump so that it was separated from the cross-suture by cervical muscles and was remote from the suture. Even then, however, some hypoglossal fibres were able

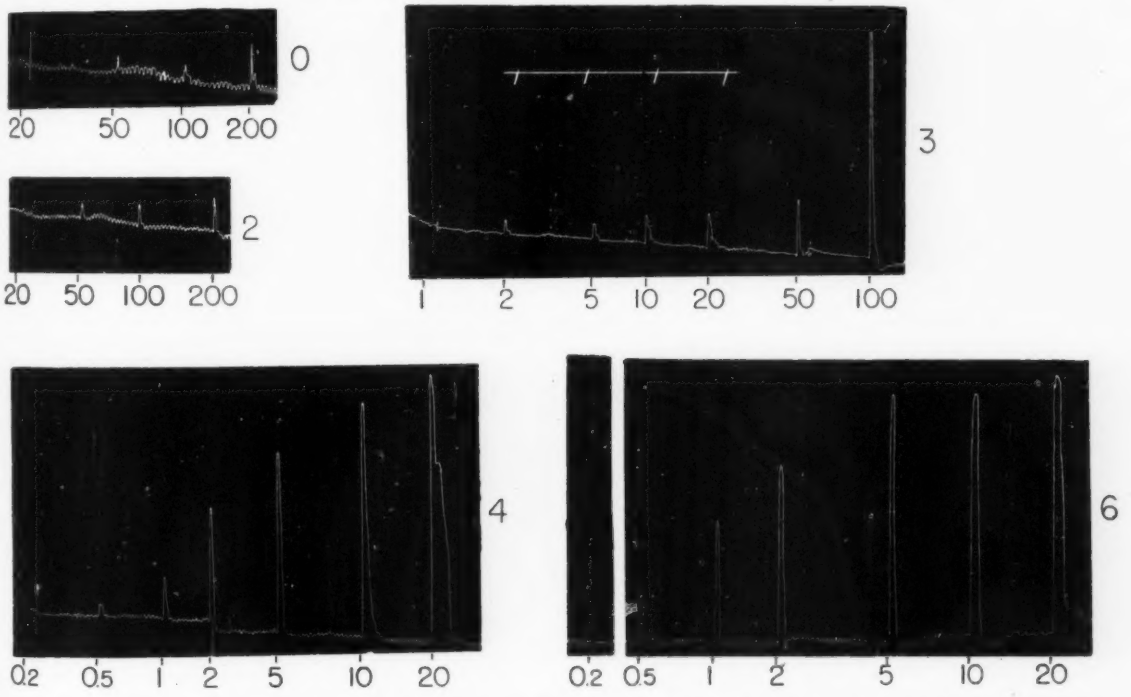

FIG. 1.—The sensitivity of the tongue muscles to acetylcholine before (**0**) and **2**, **3**, **4**, and **6** days after section of the right hypoglossal nerve. The numerals below each tracing give the doses of acetylcholine (in μg./kg.) injected intracardially. Time (on day 3), min.

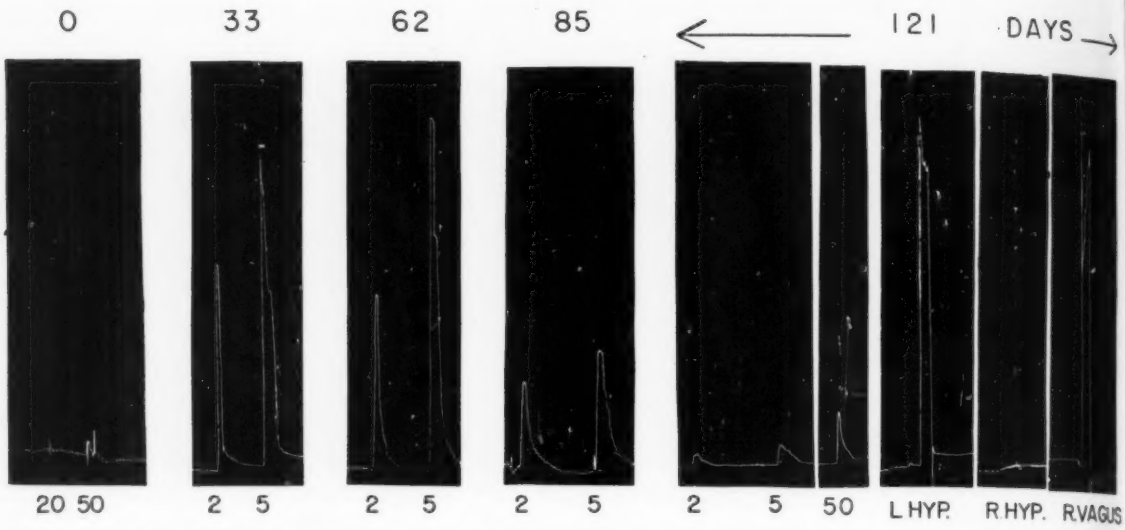

FIG. 2.—Records of sensitivity as in Fig. 1 before **(0)** and **33, 62, 85,** and **121** days after vagus-hypoglossal anastomosis. The doses of acetylcholine used to estimate the sensitivity are given as numerals below the tracings in μg./kg. **L.HYP.**=stimulation of the left, **R.HYP.** of the right hypoglossal, **R.VAGUS** of the right vagus for 10 sec., frequency 20/sec.

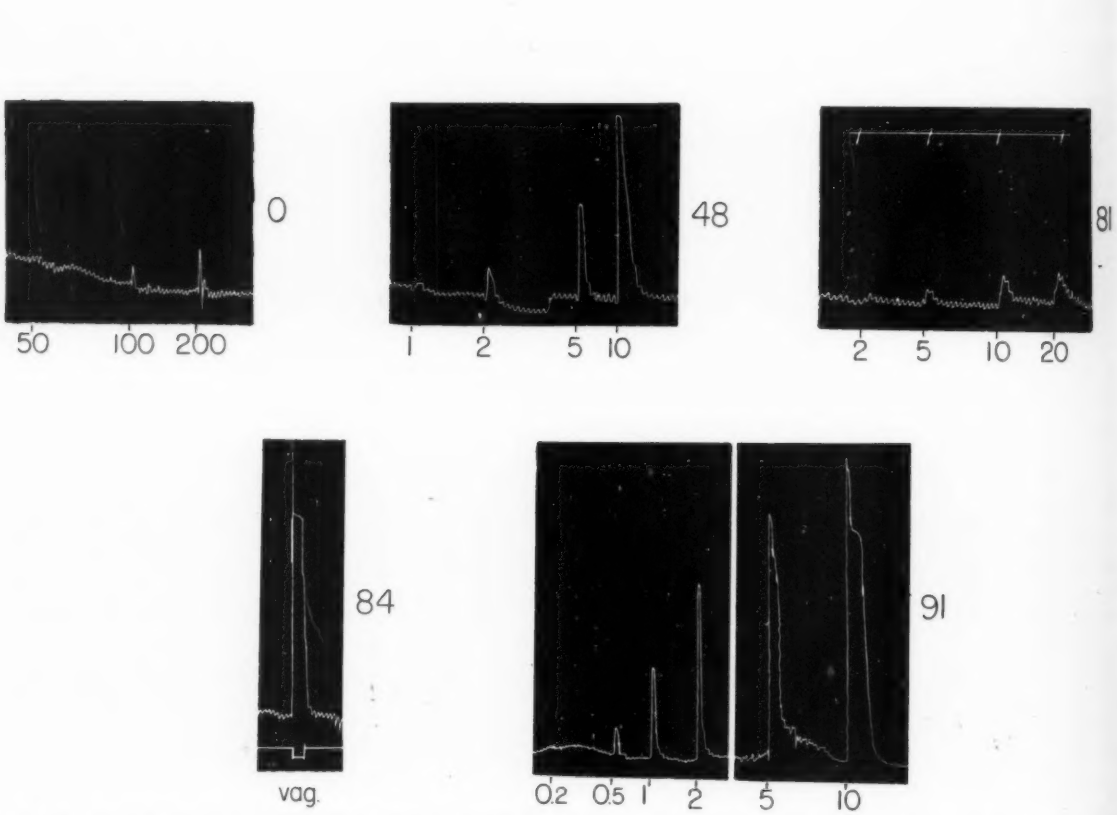

FIG. 3.—Records of sensitivity as in Fig. 1 before **(0)** and **48, 81, 84,** and **91** days after vagus-hypoglossal anastomosis. At **vag.** the right vagus was cut at the base of the skull and stimulated for 10 sec. The numerals below indicate the doses of acetylcholine in μg./kg. Time, min.

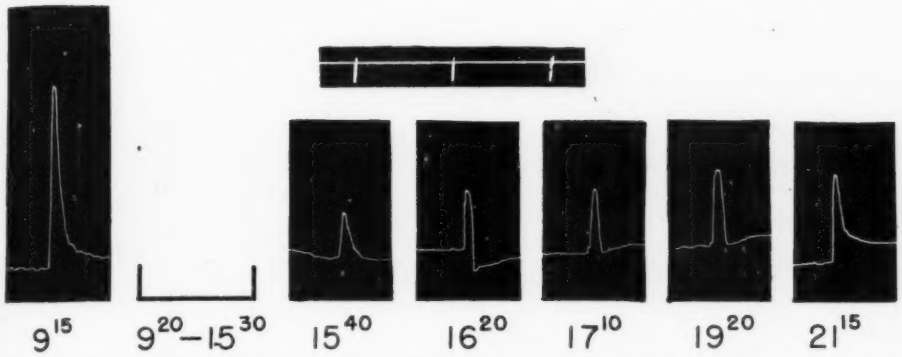

FIG. 4.—The muscles of the tongue had been sensitized by previous section of the hypoglossal nerve, and at 9^{15} the sensitivity to 5 μg./kg. of acetylcholine was high. Acetylcholine, 50 μg./kg., was injected intravenously every 5 min. between 9^{20}–15^{30}, causing strong contractions each time. Each of the following contractions is caused by 5 μg./kg. of acetylcholine, injected intracardially at the time given below the tracings. Time, min.

to find their way to the tongue in some experiments. There were in fact only three experiments in which electrical stimulation of the right hypoglossus had no effect whatsoever on the tongue. Fig. 2 shows one such experiment in which the sensitivity was very high even two months after the cross-suture had been made. After 85 days the sensitivity had markedly decreased and after 121 days it was even less. Stimulation of the right vagus caused a contraction similar to that elicited through the left hypoglossal whereas stimulation of the right hypoglossal had no effect.

The effect of vagal stimulation was abolished by decamethonium and by gallamine in doses which were of the same order of magnitude as those required to abolish the contractions caused by stimulation of the left hypoglossal.

In view of the great tendency to re-innervation from the hypoglossal nerve, it seemed important to ascertain that the decrease in sensitivity observed in the cross-suture experiment was really due to re-innervation from the vagus nerve. In a typical experiment illustrated in Fig. 3, the sensitivity was not tested until 48 days after the vagus-hypoglossal anastomosis when it was found to be high. After 81 days it had greatly decreased. Three days later the vagus nerve was cut as far centrally as possible, using hexobarbitone anaesthesia. Stimulation of the peripheral stump was found to cause contraction of the tongue. The cat was allowed to recover and a week later the sensitivity was found to be very high, even higher than in the experiment after the first denervation, probably because some re-innervation had already taken place at that stage.

In the two experiments in which the chorda-lingual nerve was sewn to the hypoglossal, stimulation of the former nerve was found to give a strong contraction when the sensitivity had declined. In the vagus-hypoglossal suture experiments, however, no contraction of the tongue was obtained when the chorda-lingual nerve was stimulated in the acute experiment, namely the Philipeaux-Vulpian phenomenon was not observed when the tongue had been re-innervated from the vagus nerve.

Treatment with Acetylcholine

Repeated injections of acetylcholine over a period of 6 hr. were found to diminish the supersensitivity caused by denervation. Fig. 4 shows the responses of the tongue muscles to a standard dose of acetylcholine, 5 μg./kg., given intracardially. Ten minutes after the end of the treatment with acetylcholine the sensitivity was markedly decreased, and estimations of the sensitivity about once an hour showed that only a slight recovery took place in the course of nearly 6 hr.

DISCUSSION

As in the submaxillary gland, the high sensitivity to chemical agents created by denervation in the muscles of the tongue is reduced when the structure becomes re-innervated by some other nerve than its own; and the experiments on the tongue have the advantage that there is no synapse in the nervous pathway.

Treatment of the decentralized gland with secretory agents for some days abolishes the supersensitivity. It seemed difficult, for technical reasons, to reproduce similar experiments on skeletal muscle. Since, however, the time course for the sensitization of the tongue muscles was found to be much shorter than that of the gland

it appeared possible that the same might apply to the "desensitization" process, and an attempt was made to "desensitize" the tongue muscles in the course of an acute experiment. The supersensitivity of the muscles was markedly reduced, although not abolished, by often repeated intravenous injections of acetylcholine during 6 hr., and the "desensitization" persisted for many hours.

In a normally innervated salivary gland, a supersensitivity resembling that caused by denervation can be produced by long-continued treatment with an anti-parasympathetic or a ganglion blocking agent (Emmelin and Muren, 1950; Emmelin, 1959). No attempts were made in the present investigation to keep a cat curarized for the days necessary for a possible sensitization. In the case of the submaxillary gland, it proved possible to sensitize with hexamethonium in cross-suture experiments, because the new synapse was extremely susceptible to the blocking agent. It was hoped that in the cross-suture experiments with skeletal muscle, decamethonium or gallamine would prove to be particularly active, so that the muscles of the tongue could be kept curarized by doses which did not affect the respiratory muscles. This was, however, not found to be the case.

Technical assistance was given by Miss Ursula Delfs.

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Brit. J. Pharmacol. (1959), **14**, 239.

NICOTINE AND THE EFFECT OF ANTISYMPATHOMIMETIC AGENTS ON THE AORTA OF THE RABBIT

BY

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The responses of strips of rabbit aorta to almost maximal doses of nicotine were less readily antagonized by five antisympathomimetic agents than were comparable responses to noradrenaline. The effect was most marked with dibenamine, ergotamine, and tolazoline: approximately twice the dose of noradrenaline was required to match the test dose of nicotine after treatment with the antagonists. Dose/response curves for nicotine before and after phentolamine 10^{-7} indicate that the phenomenon may be reversed with low doses of nicotine and that the release of noradrenaline by nicotine within the tissues is probably a graded response. The pattern of nicotine/phentolamine antagonism in this preparation is consistent with the view that nicotine acts indirectly by releasing a noradrenaline-like substance, and the difficulty found in antagonizing responses to nicotine with antisympathomimetic agents is probably similar to that responsible for failure of atropine to block some parasympathomimetic responses to nicotine.

It is well known that the effects of sympathetic nerve stimulation in various organs are less readily opposed by antisympathomimetic agents than are the effects of circulating adrenaline. This has been demonstrated for piperoxane hydrochloride by Morison and Lissak (1938) using the cat salivary gland as a test object, for tolazoline by Chess and Yonkman (1946) using the cat salivary gland and splanchnic vascular bed, and for dibenamine on the cat nictitating membrane by Nickerson and Nomaguchi (1948). Bacq and Fredericq (1935) demonstrated differences between benzodioxane blocking agents in this respect; piperoxane was found to be much less effective than compound 883F against effects produced by nerve stimulation. Bovet and Simon (1937) extended these observations, showing that in several series of benzodioxane derivatives increase in the size of the N radical brought about a transition from the 883F to the piperoxane type of compound. Apart from these observations, no direct comparisons of antisympathomimetic agents against effects of nerve stimulation appear to have been made.

The local vasoconstrictor action of nicotine is probably due to the stimulation of sympathetic nerve endings or ganglion cells in the walls of blood vessels (Hilton, 1954). Kottegoda (1953) and Burn and Rand (1958) have shown that this action of nicotine is due to the release of a noradrenaline-like substance and have discussed the question of its release. The responses to nicotine of isolated vascular smooth muscle might therefore be expected to be rather more difficult to antagonize with antisympathomimetic agents, and the relative effectiveness of such agents against these responses might be expected to resemble their effectiveness against that which follows stimulation of nerves.

METHOD

Spiral strips of aorta, 5 cm. long and 2 mm. wide, were prepared as described by Furchgott and Bhadrakom (1953) from rabbits weighing 2 to 2.5 kg. The strips were suspended in oxygenated Ringer-Locke solution in a 50 ml. bath at 37° and movements recorded with a frontal writing lever (magnification $\times 10$, tension 2 g.). The strips were allowed to relax for 1½ hr. before drugs were added.

RESULTS

There were two series of experiments. In the first, the response to a standard dose of nicotine was matched by a response to noradrenaline before and after addition of a blocking agent. In the second, a dose/response curve was plotted for nicotine before and after addition of phentolamine 10^{-7}, to describe more completely the antagonism between a typical antisympathomimetic agent and nicotine.

Comparison of the Effects of Antagonists against Responses to Nicotine and Noradrenaline

Five antisympathomimetic agents were tested. For each, six strips of aorta from three different rabbits were used. Responses to nicotine and noradrenaline were recorded alternately at 30 min. intervals. The noradrenaline doses were added in 4 successive steps at 2.5 min. intervals to give a dose/response curve. Before the addition of the blocking agent, the response to a standard dose of nicotine (3×10^{-5}) was matched twice by a response to noradrenaline. One hr. after the addition of the blocking agent, the amount of noradrenaline required to match the response to the standard dose of nicotine was again determined twice in the presence of the blocking agent. (The aorta was exposed to dibenamine for 20 min., then washed for 40 min. before testing.) The same tests were carried out on a control series of six strips, but with no antagonist present.

For each agent, results from all six strips were combined to estimate the noradrenaline equivalents before and after addition of the drug (Table I).

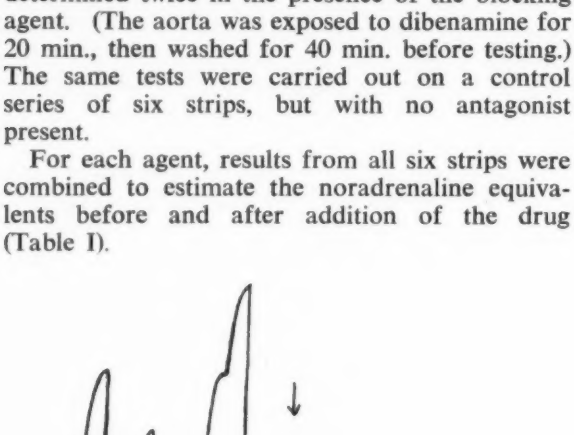

FIG. 1.—Effect of dibenamine on the responses to nicotine and noradrenaline of spiral strips of rabbit aorta. At **A**, successive doses of (−)-noradrenaline bitartrate (10^{-6}, 3×10^{-6}, 10^{-7}, and 3×10^{-7}) were added at 2.5 min. intervals. At **B**, nicotine hydrogen tartrate (3×10^{-5}) was added. The numerals give the time in min. The preparation was treated (at the arrow pointing downwards) with dibenamine hydrochloride 3×10^{-7} from 100 to 120 min. The response to nicotine was less readily antagonized by dibenamine than was a comparable response to noradrenaline. Numerals, time in min.

The responses to both noradrenaline and nicotine were reduced by the concentrations of blocking agents used, but the responses to nicotine were less affected than were those to noradrenaline. Thus, after dibenamine, the mean response to nicotine was 1.8 times greater than the response to noradrenaline which initially matched it, and approximately twice the dose of noradrenaline was required to match the test dose of nicotine. The result of a typical experiment on a single strip is shown in Fig. 1.

Nicotine/Phentolamine Antagonism

Dose/response curves were determined for nicotine in eight control strips, and in eight strips treated with phentolamine (10^{-7}) (Fig. 2). Three strips were used each day. In each group of eight, four strips received 0.25, 1, 4, and 16×10^{-5} of nicotine, and four received 0.5, 2, 8, and 32×10^{-5}.

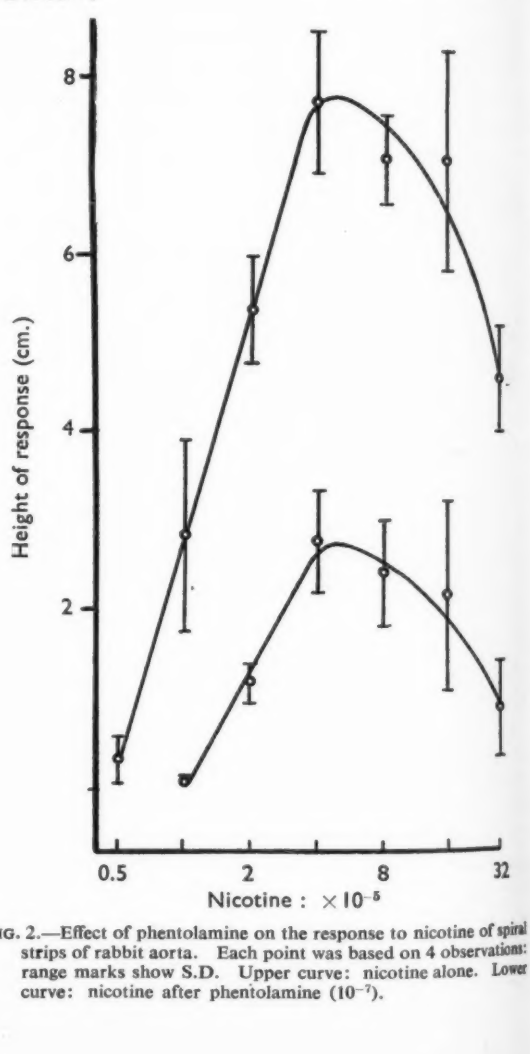

FIG. 2.—Effect of phentolamine on the response to nicotine of spiral strips of rabbit aorta. Each point was based on 4 observations: range marks show S.D. Upper curve: nicotine alone. Lower curve: nicotine after phentolamine (10^{-7}).

TABLE I

EFFECT OF ANTISYMPATHOMIMETIC AGENTS ON THE AMOUNT OF NORADRENALINE REQUIRED TO MATCH THE RESPONSE TO A TEST DOSE OF NICOTINE IN ISOLATED STRIPS FROM RABBIT AORTA

x, the ratio between doses of noradrenaline producing equivalent effects before and after the addition of antagonist, was estimated graphically from the shift of the noradrenaline dose/response curve. Mean results from 6 strips of aorta in parentheses, 95% limits (Finney, 1952, Chapter 4), are given.

Antagonist	Conc.	Noradrenaline Dose Ratio x	Noradrenaline ($\times 10^{-8}$) Required to Match Response to Nicotine 3×10^{-5}, 5% Probability Limits		Ratio B/A
			Before (A)	After (B)	
Dibenamine	3×10^{-7} for 20 min.	8	8·18 (6·29–11·2)	17·8 (13·3–23·8)	2·17
Ergotamine tartrate ..	10^{-7}	13	9·62 (7·25–13·9)	20·8 (13·7–34·1)	2·17
Tolazoline	10^{-6}	6	12·6 (8·49–23·8)	24·8 (17·1–40·0)	1·96
Phentolamine	10^{-7}	6	8·67 (5·99–14·4)	13·6 (10·5–17·3)	1·57
Piperoxane	10^{-6}	10	6·55 (4·81–9·23)	8·62 (5·14–12·1)	1·32
None			17·75 (12·7–25·0)	12·6 (9·01–16·6)	0·71

DISCUSSION

The results described indicate that the response to a near maximal dose of nicotine (3×10^{-5}) was less readily antagonized by antisympathomimetic agents than was a comparable response to noradrenaline. This difference was most marked with dibenamine and ergotamine, where approximately twice the dose of noradrenaline was required to match the test dose of nicotine after treatment with the blocking agents. The other agents examined behaved similarly to some extent, while the control series showed a drift in the opposite direction. Further experiments would be necessary to determine whether these differences between antisympathomimetic agents are real, but it is notable that piperoxane differentiated nicotine and noradrenaline responses least effectively, despite its reputed ineffectiveness against nerve responses.

It was not expected that there would be marked difficulty in blocking the responses to nicotine. Kottegoda (1953) reversed the response to nicotine in the perfused rabbit ear with tolazoline 2×10^{-5}, and Hilton (1954) used phentolamine to demonstrate that nicotine acted sympathomimetically on the blood flow in cat muscle. The concentration of tolazoline used by Kottegoda (1953) was high: the above results show that it would reduce the sensitivity of the preparation to noradrenaline 100-fold.

Haimovici (1948) reported that the responses to nicotine in perfused frog hindquarters were very difficult to block with dibenamine. He claimed that perfusion with dibenamine (4 to 40 mg./ml.) had no effect on the response to nicotine but selectively abolished the response to adrenaline, and used this as evidence that nicotine has a direct vasoconstrictor action in this preparation. The resistance seen in the rabbit aorta was obviously much less marked. The atropine resistance shown by nicotine responses in the isolated rabbit ileum is a similar phenomenon, although more marked. Ambache and Edwards (1951) found that atropine 10^{-4} did not abolish contractions in response to nicotine, although atropine 10^{-6} antagonized large doses of acetylcholine. In the ileum nicotine acts parasympathomimetically (Ambache, 1955), and here, too, the effects of nerve stimulation are antagonized with difficulty. In both cases there are two possible explanations: first that nicotine may liberate high local concentrations of the transmitter within the tissues; second that the transmitter may be released behind a diffusion barrier which is impermeable to both the antagonist and the transmitter substance (see Nickerson and Nomaguchi, 1948). In an isolated organ it is possible that the thickness of the tissue itself might act as a diffusion barrier, nicotine alone of the drugs applied being capable of penetrating throughout the tissue and the others acting only on the surface layers. This seems improbable since the phenomenon was seen for nerve effects *in situ*.

The dose/response curves for nicotine before and after phentolamine 10^{-7} show that the response to nicotine was readily surmountable, as one would expect if it acted by liberating a limited amount of noradrenaline within the tissues. The inhibitory action of large doses of nicotine appeared to be little affected by phentolamine. In the presence of phentolamine 10^{-7} large doses of nicotine (32×10^{-5}) produce a biphasic response in the strips, contraction being followed by relaxation beyond the resting length. Low concentrations of nicotine were apparently not inhibitory, the response to nicotine 10^{-5} (about one-third of the maximal response) being completely abolished by phentolamine 10^{-7}, but the response was not reversed.

Phentolamine 10^{-7} antagonized responses to low doses of nicotine with unexpected ease (Fig. 2). Low doses of nicotine must liberate a uniformly low concentration of noradrenaline within the tissues rather than a high concentration at only a fraction of the muscle fibres, that is to say, the response of the nervous structures stimulated by nicotine must be graded rather than all-or-none. The ease with which low doses of nicotine were antagonized suggests that the amount of difficulty experienced in blocking the response to nicotine may depend on the dose of nicotine. Responses to low doses of nicotine may be more easily blocked than equivalent responses to noradrenaline, although a direct comparison would be required to determine this.

I wish to express my gratitude to Professor A. D. Macdonald for his advice and encouragement throughout this work which was carried out during the tenure of a Benger Research Fellowship.

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CENTRAL EFFECTS PRODUCED BY INJECTION OF 48/80 INTO THE CEREBRAL VENTRICLES OF MICE

BY

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Injections of 20 to 30 μg. of 48/80 into the cerebral ventricular spaces of mice produced a condition of motor excitement termed " delirium ambulatorium." This condition was aggravated by previous intraperitoneal injection of tranquillizers, atropine, and promethazine, not affected by previous injection of mepyramine and abolished by a previous injection of pentobarbitone. When an intraperitoneal injection of strychnine was followed by an intraventricular injection of 48/80, the strychnine convulsions became more violent, but the pattern of delirium ambulatorium was not produced.

Compound 48/80 has powerful histamine releasing properties (Paton, 1951; Feldberg and Paton, 1951; Feldberg and Talesnik, 1953), and under certain conditions also releases 5-hydroxytryptamine from mast cells. These disintegrate under the influence of 48/80 (Bhattacharya and Lewis, 1956; Parratt and West, 1957). However, its high toxicity and lethal effects cannot be attributed entirely to the systemic effects of released histamine or 5-hydroxytryptamine since the signs elicited by giving 48/80 to guinea-pigs are not typical of histamine poisoning (Mota and Vugman, 1956; Mota, 1957), and the amounts of 5-hydroxytryptamine released are too small to produce lethal effects in most species. Some of the toxic effects of 48/80 in guinea-pigs and rats may result from stimulation of the central nervous system. This suggestion is borne out by the following experiments in which 48/80 was injected intracerebrally of which a preliminary report was made at the tenth annual meeting of the Brazilian Society for the Advancement of Science in São Paulo, July 6 to 12, 1958.

METHODS

The experiments were performed on mice weighing between 18 and 22 g. Mice were chosen because they are relatively insensitive to the systemic effects of histamine. The 48/80 was injected in saline solution into the cerebral ventricles through the intact skull 2 to 3 mm. in front of the line joining the anterior base of the ears using a fine 26 gauge needle 0.5 cm. long according to the method described by Haley (1957) and Haley and McCormick (1957). The volume of injection was between 0.02 and 0.09 ml.

In order to find out if the effects of intracerebral 48/80 were influenced by other drugs, these were injected intraperitoneally in a volume of 0.1 to 0.2 ml. of saline solution some time before the intracerebral injection of 48/80. The time interval between the two injections was between 20 and 45 min., except with reserpine which was given 2.5 hr. before 48/80. Perphenazine (Trilafon: 1(2 - hydroxyethyl) - 4,3(2 - chlor-10 phenylthiazyl-propyl) piperazine) was also used.

RESULTS

Within a few minutes an intracerebral injection of 20 to 30 μg. of 48/80 led to a condition of deep depression. This lasted for 2 to 3 min., then the mouse suddenly started to run forward with well-co-ordinated but extremely quick movements. It either moved in a straight line or in wide circles. Convulsions were rarely seen at this early stage. The mouse seemed to have lost all sense of boundaries. When it jumped from a table it continued its forward course as soon as it reached the floor, until it hit an obstacle, and there were often periods in which the mouse continued to roll over on its side for many seconds. This " psychotic " behaviour has been termed " delirium ambulatorium." It ended usually after 5 to 10 min. by death or exhaustion or by a prolonged hyperkinetic state of jerky movements or of clonic convulsions. 80% of the injected animals developed all the signs of the delirium ambulatorium and half of these remained in a hyperkinetic state for many hours. In two mice signs of delirium ambulatorium with periods of rolling over continued for 24 hr. Occasionally animals died with symptoms of respiratory arrest

after 5 to 10 min. of intense running activity; this happened more frequently with higher doses (40 μg.) of 48/80.

Effects of Drugs on the Delirium Ambulatorium and Mortality Produced by 48/80

The results are summarized in Table I. Each drug was tested in groups of five mice to find out if it influenced the delirium ambulatorium and the mortality which was about 10% following the injection of 20 to 30 μg. of 48/80 alone.

When 48/80 was injected in mice during pentobarbitone anaesthesia, it greatly prolonged the sleeping time due to the barbiturate. The only stimulating effects produced by the injection of 48/80 in the anaesthetized animals were an increase in depth and rate of respiration and an increased general reflex excitability, particularly to touch and noise.

When given together with either promethazine or chlorpromazine before the injection of 48/80, pentobarbitone prevented not only the occurrence of the delirium ambulatorium but also the high mortality of mice treated with either promethazine or chlorpromazine before the administration of 48/80.

Table I shows that the two antihistamine drugs tested, promethazine and mepyramine, influenced the effects of the intracerebral injection of 48/80 in different ways. Promethazine strongly enhanced the delirium ambulatorium and increased the mortality to 60% whereas mepyramine influenced neither the delirium ambulatorium nor the mortality. This difference is probably not due to the fact that promethazine has a more potent antihistaminic effect than mepyramine. The difference may well be due to the fact that promethazine has also atropine-like properties, since atropine also increased the delirium ambulatorium and the mortality caused by 48/80 injections. The enhancing effect of perphenazine, another phenothiazine derivative, may also result from its atropine-like properties.

In the experiments with strychnine, a dose was chosen which was not lethal and which produced only mild convulsions. The subsequent injection of 48/80 did not alter the pattern of the strychnine convulsions; they became violent but the condition did not revert to the pattern of delirium ambulatorium.

It is interesting to note that the powerful tranquillizers, chlorpromazine, promethazine, and perphenazine, increase the delirium ambulatorium and lethality of the 48/80 injections and that reserpine and Serpasol (Ciba) have a similar effect in doses which, when given alone, would produce a tranquillizing effect.

TABLE I

EFFECTS OF DRUGS INJECTED INTRAPERITONEALLY ON THE DELIRIUM AMBULATORIUM AND MORTALITY IN MICE PRODUCED BY SUBSEQUENT INTRACEREBRAL INJECTIONS OF 20 TO 30 μG. OF 48/80

All doses in mg./kg. body weight except reserpine and strychnine (mg./animal).

Drug Injected Before 48/80		Effect on Delirium Ambulatorium	% Mortality
Reserpine	2	Slight increase	0
Serpasol	5	Large ,,	80
Chlorpromazine	12	Slight ,,	40
	25	,, ,,	20
Atropine sulphate	1·25	Large ,,	60
Mepyramine maleate	50	No effect	0
Perphenazine	5	Large increase	100
Strychnine	0·02	Tonic convulsions	60
Promethazine hydrochloride	12·5	Large increase	80
Pentobarbitone	30–50	Abolition	0
,, plus			
(a) Promethazine	12·5	,,	0
(b) Chlorpromazine	12	,,	0
(c) Mepyramine	50	,,	0

DISCUSSION

The peculiar motor excitement produced in mice by intracerebral injections of 48/80 and termed delirium ambulatorium is probably due to stimulation of subcortical structures lining the ventricular spaces. It is, at present, not possible to state whether the effect is produced indirectly by the release of histamine, 5-hydroxytryptamine or some unknown substance, or whether it is the result of a direct stimulating action of 48/80 itself. It is interesting and relevant that 48/80 is not the only histamine liberator which on intracerebral injection produces the peculiar motor excitement; tubocurarine, which is also a histamine liberator, produces on injection into the cerebral ventricles of cats and mice a pattern of motor excitement which resembles in many features the delirium ambulatorium produced by 48/80 (Feldberg and Sherwood, 1954; Haley, 1957).

The fact that intracerebral injections of histamine or 5-hydroxytryptamine, even in large doses, do not produce in mice motor effects resembling delirium ambulatorium (Haley, 1957; Rocha e Silva, unpublished observations) does not exclude the possibility that these substances have such an action when released close to the reacting structures in the brain. If the central effect of 48/80 were due to release of histamine, it could not be caused by an action of histamine on the cerebral vessels because the effect persisted and was even increased after the injection of antihistaminic drugs which are known to reduce

greatly the vascular effects of released histamine (Virno, Gertner, and Bovet, 1956). On the other hand, since histamine releases catechol amines from the suprarenals, an analogous effect on these substances in the brain may be another way in which released histamine could produce central effects.

If the central effects of 48/80 result from the release of 5-hydroxytryptamine or catechol amines, the influence of reserpine which somewhat increases the delirium ambulatorium due to 48/80 is of interest, although this effect can be interpreted against as well as in favour of such an indirect mode of action of 48/80. Reserpine does not produce delirium ambulatorium although it reduces the 5-hydroxytryptamine and catechol amine content of the brain. This might seem to oppose the idea that these amines are involved in the production of the delirium ambulatorium. However, the fact that reserpine requires several hours for its action, whereas the effect of 48/80 occurs within a few minutes, might account for the difference, and the augmentation by reserpine of the delirium ambulatorium produced by 48/80 might be interpreted as a summation of a chronic with an acute release of these amines.

The finding that intracerebral 48/80 prolongs the sleeping time of pentobarbitone anaesthesia and increases the toxicity to strychnine shows that 48/80 has some action in common with tranquillizers and atropine-like substances, since these also prolong barbiturate sleep and increase strychnine toxicity (Sherman, 1956; Berger, Hendley and Lynes, 1956; Berger, 1957). This common mode of action might also explain why these substances aggravate the delirium ambulatorium and increase the mortality of intracerebral 48/80.

These facts suggest that 48/80, although it stimulates and produces delirium, can provide a useful tool in the study of the mechanism of action of tranquillizers. The potentiation of the effects of 48/80 by chlorpromazine, reserpine, and promethazine indicates that the tranquillizing activity is a compromise between two opposing activities, stimulation and sedation, upon different sites in the brain.

I should like to make grateful acknowledgment to Mr. H. Werneck for his technical help during the experiments. Crystalline reserpine was kindly supplied by Dr. H. J. Bein (Basle) and Trilafon (perphenazine) by Schering (Brazil).

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CARDIOVASCULAR EFFECTS OF INTRAVENOUS INFUSIONS OF 5-HYDROXYTRYPTAMINE IN MAN

BY

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Intravenous infusions of 5-hydroxytryptamine creatinine sulphate in doses of 1, 2, and 3 mg./min. of the salt into 42 normal subjects caused a dilator response of the forearm vessels, consisting of an initial transient increase in flow usually followed by a smaller but sustained increase. A fall in forearm flow was not seen. Heart rate was invariably increased and the increase preceded other changes by 10 to 20 sec. suggesting a direct effect on the heart. The sustained increase in flow probably represents a balance between the direct constrictor action of the drug and a secondary dilator effect. The blood pressure response was variable and it appeared unlikely that it was responsible for more than a small part of the forearm flow changes. Two subjects appeared to be abnormally sensitive to the drug.

5-Hydroxytryptamine has been shown to be a powerful vasoconstrictor substance, and its direct action on the smooth muscle of blood vessels has been demonstrated in isolated preparations, in animals and in man (Reid and Rand, 1951 ; Page, 1942 ; Reid, 1952 ; Roddie, Shepherd and Whelan, 1955). However, intra-arterial injection of 5-hydroxytryptamine into a limb, although it reduces the total blood flow, results also in a flushing of the skin, indicating dilatation of small vessels ; a similar flushing is seen in patients with carcinoid tumours when large amounts of 5-hydroxytryptamine are circulating in the blood stream (Roddie *et al.*, 1955 ; Thorson, Biörck, Björkman, and Waldenström, 1954). Furthermore, the variability of the blood pressure changes seen on intravenous infusions of 5-hydroxytryptamine (Page and McCubbin, 1953) suggests that it does not behave as a general overall vasoconstrictor substance.

In order to determine to what extent the constrictor action of 5-hydroxytryptamine is involved in the vascular responses when it is circulating generally in the blood stream, continuous intravenous infusions of the drug were given to normal subjects and observations made of the forearm blood flow, arterial pressure, and heart rate.

METHODS

The subjects were ourselves, our colleagues, and volunteer medical students. Ages ranged from 19 to 35 years. Experiments were carried out at a laboratory temperature of $22 \pm 2°$. The subjects were lightly clothed and rested recumbent on a couch for approximately 30 min. before recordings commenced. Forearm blood flow was measured using the technique of venous occlusion plethysmography, water-filled plethysmographs (Greenfield, 1954) at 34 to 35° being applied to one or both forearms. Blood pressure was recorded by means of a capacitance manometer from a needle in the brachial artery. Saline (0.9%) was given by means of a mechanically driven syringe through a needle or a nylon catheter inserted into an antecubital vein and infused throughout the experiment at a constant rate of 4 ml./min. The saline infusion was replaced for a period of 10 min. when required by a solution of 5-hydroxytryptamine creatinine sulphate made up in 0.9% saline. Doses are expressed throughout as mg./min. of the salt. If the responses and symptoms produced by 1 mg./min. were slight or moderate in degree, a second infusion of 2 mg./min. and, in some cases, a further infusion of 3 mg./min. was given with intervals of 5 to 10 min. between infusions.

RESULTS

5-Hydroxytryptamine in a dose of 1 mg./min. was infused on 28 occasions in 25 subjects ; 14 of these also received 2 mg./min. and 9 received 3 mg./min. In all cases, forearm blood flow was measured, and in 15 experiments arterial blood pressure was recorded in addition. Pulse rates were obtained from the blood pressure record or the plethysmogram in 41 experiments. In four subjects the electrocardiogram was also recorded.

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Forearm Blood Flow. — The blood flow responses varied in different subjects. The common pattern of response is shown in the upper graph in Fig. 1. There was an initial transient increase in flow commencing within 1 to 1.5 min. of the beginning of the infusion and followed by an elevation in flow sustained through the remainder of the infusion, the flow gradually returning to the resting level over a period of 5 to 10 min. after the infusion ceased.

The transient increase in flow was present in 16 of 28 experiments with 1 mg./min.; in 10 of 14 experiments with 2 mg./min., and 8 of the 9 experiments with 3 mg./min.

The sustained increase in flow was present in 20 of the 28 experiments with 1 mg./min.; 13 of the 14 with 2 mg./min., and all 9 of those with 3 mg./min.

In three subjects there was neither a transient nor a sustained increase in flow in the forearm during the 5-hydroxytryptamine infusion. In only one of the total of 51 infusions was a fall in blood flow observed.

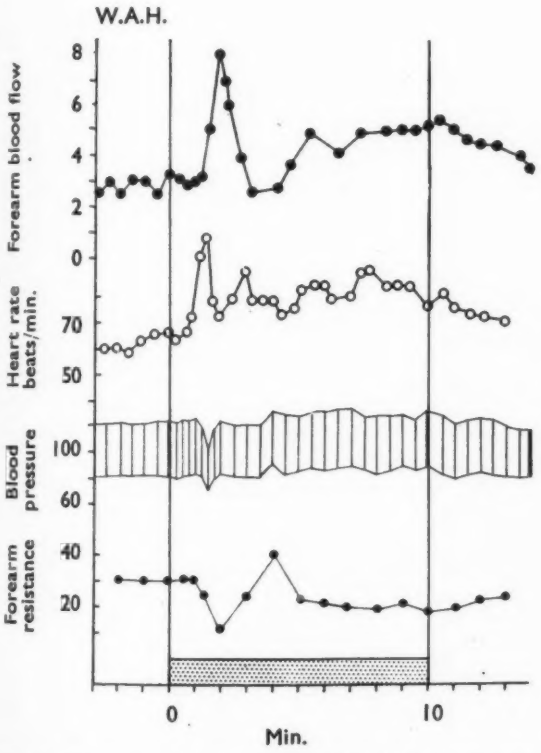

FIG. 1.—The responses of the forearm blood flow (●), heart rate (O), blood pressure, and forearm vascular resistance (•) of subject W. A. H. to 5-hydroxytryptamine creatinine sulphate administered intravenously in a dose of 2 mg./min. for 10 min. during the period indicated by the dotted area. The forearm resistance (mean pressure in mm.Hg/forearm flow in ml./100 ml./min.) is expressed in arbitrary units, blood pressure in mm. Hg, heart rate in beats/min. and forearm blood flow in ml./100 ml./min.

Nerve-Blocked Forearm.—In two experiments on two different subjects the response of the forearm blood blow to intravenous infusion (1 mg./min. of 5-hydroxytryptamine for 10 min.) was observed before and after the nerves to the forearm had been blocked by infiltration with 1% lignocaine (Whelan, 1952). The result of one of these experiments is shown in Fig. 2. The forearm flow was elevated following the nerve block as a consequence of release of vasoconstrictor tone, but the pattern of response to 5-hydroxytryptamine was not altered, and a transient, followed by a sustained, increase in flow still occurred.

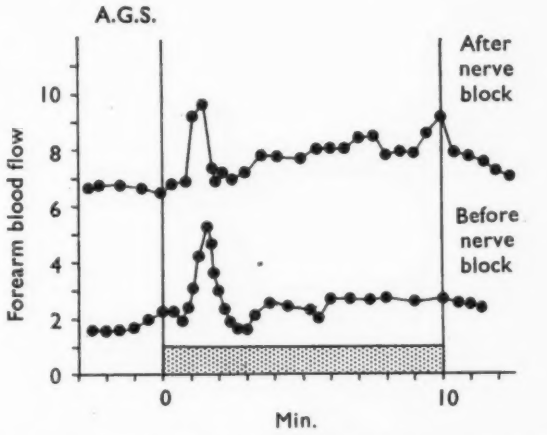

FIG. 2.—The response of the blood flow through the left forearm of subject A. G. S. to an intravenous infusion of 1 mg./min. of 5-hydroxytryptamine indicated by the dotted area before (lower curve) and after (upper curve) block of the nerves to the forearm with local anaesthetic. See legend to Fig. 1 for explanation of forearm blood flow.

Pulse Rate.—On all of the 41 occasions on which pulse rate records were obtained there was an increase during infusion of 5-hydroxytryptamine at each of the three concentrations used. The increase in heart rate commenced within 1 to 1.5 min. of the commencement of the infusion, and it invariably preceded, by 10 to 15 sec., the initial flow increase in the forearm. In 38 experiments, the pattern of the heart rate change was similar to the forearm flow changes in that there was an initial transient increase of 10 to 40 beats/min. followed by a sustained tachycardia of 5 to 30 beats/min. (Fig. 1), the larger doses having the more marked effect. In three experiments, changes in pulse rate were observed to be present but unaccompanied by any sustained increases in forearm blood flow. The results of

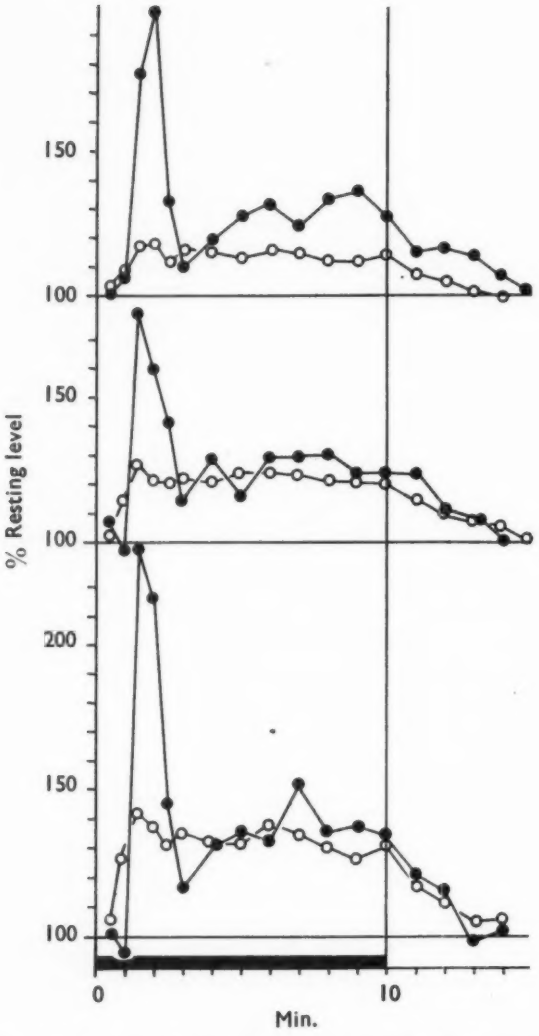

FIG. 3.—The averaged results for all experiments in which measurements of forearm blood flow and heart rate were obtained simultaneously before, during, and after intravenous infusion of 5-hydroxytryptamine indicated by the black bar. The changes in forearm flow (●) and heart rate (O) have been expressed as % of the mean of the resting values during the last 5 min. of the control period before the infusion began. Upper curves, mean of 21 experiments with a dose of 1 mg./min.; middle curves, mean of 16 experiments with a dose of 2 mg./min.; lower curves, mean of 7 experiments with a dose of 3 mg./min.

experiments in which the pulse rate and the forearm blood flow were recorded simultaneously are shown in Fig. 3.

Blood Pressure.—The blood pressure response to intravenous infusion of 5-hydroxytryptamine was variable. With 1 mg./min., in three experiments there was no change in pressure; in four there were sustained increases in mean pressure of 7, 8, 13, and 20 mm. Hg respectively. One subject showed a fall in pressure. With 2 mg./min., in three experiments there was a rise in mean pressure (6, 15, and 16 mm. Hg); in two there was no change, and in one a slight fall. With 3 mg./min. in the one experiment there was a rise in mean pressure of 13 mm. Hg.

There did not appear to be any consistent relationship between blood pressure changes and changes in forearm blood flow. In the eight subjects who showed a rise in pressure there was also an increase in flow. In five subjects there was no change in pressure, but an increase in flow occurred in four of these. Two subjects showed a fall in pressure accompanied by an increase in flow.

Electrocardiogram.—Records were obtained in seven experiments with infusions of 1, 2, and 3 mg./min. No abnormalities in conduction or rhythm were noted except a reduction in the P-R interval consistent with the tachycardia.

Signs and Symptoms.—The incidence of symptoms was found to increase with increasing doses of 5-hydroxytryptamine. Thus in only 12 of 28 experiments with 1 mg./min. were symptoms noted, while with 2 mg./min. 13 of 14 noticed symptoms and with 3 mg./min. all subjects were affected. The symptoms, in descending order of frequency of occurrence, were: pain along the course of the infused vein; heaviness in the legs and arms; pressure in the head and eyes; nausea; awareness of increased respiration; abdominal cramps; tightness in the chest. Miscellaneous symptoms which were occasionally noted were pulsation of arteries, blurred vision, sweating, excessive salivation, headache, lightness in the head, and an abnormal sense of smell. Signs noted by the observers included a malar flush and sometimes a flush of the neck occurring 2 to 3 min. after the beginning of the infusion. Erythema and tenderness along the course of the infused vein commonly occurred and often extended into the axilla. No flushing of the forearm or hand skin was apparent at any of the doses used.

The incidence of thrombosis appeared to be abnormally high in the veins infused with solutions of 5-hydroxytryptamine. In some cases this was noted at the end of the experiment, while in others on the following day the vein was found to be tender, thickened, and cord-like.

Abnormal Responses.—In two subjects the responses to intravenous 5-hydroxytryptamine were sufficiently different from the usual to warrant separate description. In the first of these (Fig. 4) forearm blood flow and brachial arterial

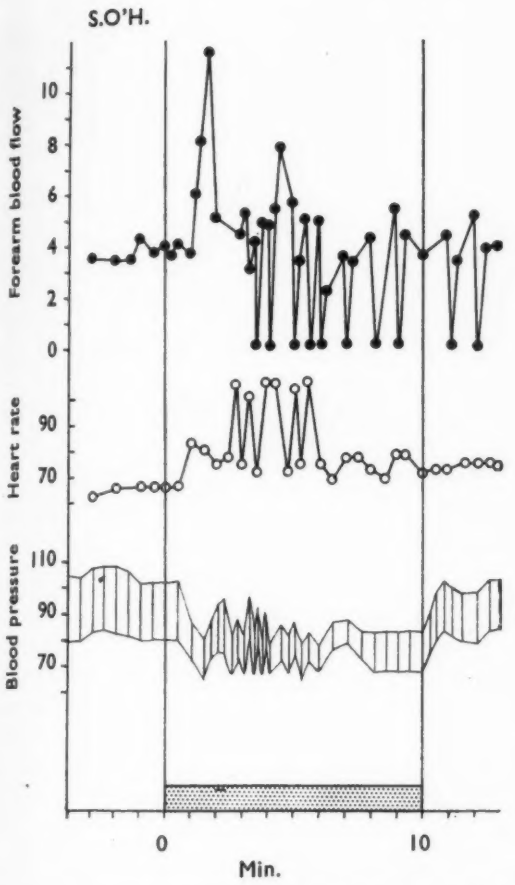

FIG. 4.—The changes in forearm blood flow (●), heart rate (O) and blood pressure in subject S. O'H. during intravenous infusion of 1 mg./min. of 5-hydroxytryptamine for 10 min. indicated by the dotted area. For explanation of ordinates, see legend to Fig. 1.

blood pressure were measured. In the first 3 min. of a 10 min. infusion of 5-hydroxytryptamine at a dose of 1 mg./min., an initial transient increase in blood flow occurred, preceded 15 to 20 sec. earlier by a rise in heart rate from 65 to 80 beats/min., and accompanied by a fall in blood pressure from the pre-infusion level of 105/80 to 80/65 mm. Hg. For the remaining 8 min. of the infusion, the blood pressure, the heart rate, and the blood flow showed a series of marked oscillations. The peaks in the blood pressure never rose above 100 mm. Hg systolic. On cessation of the infusion, the blood pressure and heart rate returned to normal levels within 1 to 2 min. though the forearm flow continued to show fluctuations. The subject had no symptoms during or after the infusion. Seven weeks later he submitted to a second infusion of the same dose. Within 2 min. of the onset of the infusion, the blood pressure fell to 75/35 mm. Hg, the heart rate rose from 72 to 100 beats/min., and the forearm flow increased fourfold. Symptoms of nausea and palpitation were present and the infusion was stopped after 1.75 min., following which the pressure and heart rate returned to normal over a period of 5 min. An electrocardiogram was taken throughout, and this showed, in addition to a shortening of the P-R interval associated with tachycardia, two isolated instances of inversion of the P wave, one just before the end of the infusion and one just after.

The second subject received 1 mg./min. of 5-hydroxytryptamine for a period of 30 min., while the forearm blood flow was being measured. The usual transient increase in flow occurred followed by a moderate sustained increase in flow. In the last 3 min. of the infusion the forearm flow rose markedly and the subject complained of nausea, pounding in the head and legs, and showed sweating and a marked pallor. The infusion was discontinued and the effects passed off over 5 min. Four weeks later a second infusion of 1 mg./min. was given to this subject. This had to be terminated at the end of 1.5 min. because of the marked symptoms of nausea, headache, dyspnoea, shivering, and sweating which developed. Recovery occurred within 5 to 10 min. of stopping the infusion.

DISCUSSION

The direct action of 5-hydroxytryptamine on the blood vessels of the limbs in man has been shown to be a complex one. When it is infused into the brachial artery at a constant rate of 1 to 16 μg./min. the blood flow through the forearm, after an initial transient dilatation, is reduced, indicating a constriction of arterioles (Roddie *et al.*, 1955); the capacity vessels, probably mainly the veins and venules, show an increase in their tone (Glover, Greenfield, Kidd, and Whelan, 1958); the capillaries are dilated as evidenced by the flushing of the skin, and, with larger doses, there is an increase in limb volume suggesting an increased capillary permeability and oedema formation (Roddie *et al.*, 1955).

The results of the present experiments demonstrate that, when 5-hydroxytryptamine was given by the intravenous route so that a concentration comparable to the intra-arterial doses mentioned above circulated generally through the body, an initial transient 3- to 4-fold increase in forearm blood flow was still seen but the subsequent vasoconstriction did not occur, being replaced in the majority of cases by an increased flow of 20 to 30% above the resting

level. This suggests that when 5-hydroxytryptamine is given intravenously some secondary dilator mechanism is in force to oppose the direct constrictor action of the drug.

The averaged results for simultaneous blood flow and heart rate measurements shown in Fig. 3 demonstrate that the heart rate response increases with increasing doses of 5-hydroxytryptamine. Such a relation was not apparent in the case of the sustained effect on forearm blood flow, which supports the suggestion that the forearm response is a resultant of two opposing effects, an indirect dilator action being countered by a direct constrictor action. If both of these effects were equally enhanced by increased doses, the balance could be maintained almost constant at the different doses.

That the dilator action is not mediated by way of the sympathetic nerves as the result of some central or reflex effect of 5-hydroxytryptamine is shown by the fact that the dilator response was not modified by blocking the nerves to the forearm.

The dilator action may in part be a result of a raised perfusion pressure in the limb as a consequence of a raised blood pressure, but is unlikely to be wholly accounted for in this way since the rise in pressure, when it occurred, was not great and was probably insufficient to account for the accompanying increase in forearm flow if a direct pressure/flow relationship was assumed. Furthermore, an increase in forearm blood flow has been observed in a number of experiments in which the blood pressure had fallen or remained unchanged.

The response of the forearm flow is in many respects similar to that seen during intravenous infusion of adrenaline (Allen, Barcroft, and Edholm, 1946) although the sustained dilatation was usually less marked than with adrenaline. It has been shown by Reid and Rand (1951) that 5-hydroxytryptamine can release adrenaline from the adrenal medulla, and it is possible that the vascular effects of 5-hydroxytryptamine in man may in part be a consequence of release of adrenaline.

The pattern of the heart rate changes is also similar to that seen with adrenaline. However, in the present experiments the rapid onset of the tachycardia after the beginning of the infusion and the fact that it invariably preceded the changes in forearm flow and blood pressure suggest that the tachycardia was caused by the direct action of 5-hydroxytryptamine on the heart and not as a consequence of adrenaline release nor of any haemodynamic reflexes due to changes in peripheral resistance. This is further supported by the fact that tachycardia was seen in a number of experiments with doses of 5-hydroxytryptamine which had no effect on the blood pressure or peripheral flow.

The abnormal responses seen in two of the subjects were suggestive of a hypersensitivity to 5-hydroxytryptamine, particularly in view of the repetition of the effects on the second infusion some weeks later. No corresponding reports of this nature have been found in the literature available to us. As a consequence of these findings, it was considered advisable in all subsequent experiments to carry out an infusion of 1 mg./min. for at least 5 min. as a preliminary to the administration of any larger dose.

The high incidence of venous thrombosis observed in this series after intravenous infusions, the pain and tenderness along the course of the vein, and the redness of the overlying skin are evidence of the local irritant properties of 5-hydroxytryptamine.

No flushing of the forearm or hand skin was apparent at any of the dose levels, and, while it was not possible to state whether the forearm vasodilatation originated in the muscles or the skin vessels, or both, it seems unlikely that it could wholly be accounted for by dilatation of skin vessels.

This work was undertaken during the tenure by C. J. S. of a National Health and Medical Research Council Fellowship. We are grateful to our colleagues and students who volunteered as subjects for these experiments. We wish to express our appreciation of the technical assistance of Mr. A. McNeil and Mr. R. Clark. 5-Hydroxytryptamine creatinine sulphate was kindly supplied by Messrs. Sandoz, Basle, Switzerland.

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PHARMACOLOGICAL METHODS OF ESTIMATING INHIBITION OF DRUG OXIDATION IN THE MOUSE

BY

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Three pharmacological techniques for measuring inhibition of "non-specific" oxidase activity in the mouse are described: (1) potentiation of pentobarbitone hypnosis, (2) potentiation of chlorpromazine hypothermia; and (3) reduction in the toxicity of octamethylpyrophosphorodiamide (schradan). Iproniazid, isoniazid, and β-diethylaminoethyl 3,3-diphenylpropylacetate (SKF 525A) gave comparable results in all three tests.

Experiments *in vitro* have shown that mammalian liver slices carry out the oxidative transformation of a wide range of drugs of unrelated chemical structure, such as pentobarbitone, pethidine, chlorpromazine, amidopyrine, ephedrine, codeine and phenacetin. The addition of β-diethylaminoethyl 3,3-diphenylpropylacetate (SKF 525A) inhibits many of these transformations. The literature concerning this "non-specific" enzyme system has recently been reviewed (Brodie, 1956; Brodie, Gillette and La Du, 1958).

β-Diethylaminoethyl 3,3-diphenylpropylacetate potentiates the hypnotic effects of pentobarbitone in mice and rats (Cook, Toner and Fellows, 1954) by reducing the rate of bio-transformation of the pentobarbitone (Axelrod, Reichenthal and Brodie, 1954). It also potentiates the hypothermic action of chlorpromazine in mice (Lessin and Parkes, 1957a). Kamm, Gillette, and Brodie (1958) have shown that chlorpromazine is metabolized by the "non-specific" oxidase into the relatively inactive chlorpromazine sulphoxide.

The same mammalian liver enzyme system can, *in vitro* and *in vivo*, convert octamethylpyrophosphorodiamide (schradan) into a highly toxic cholinesterase inhibitor (Gardiner and Kilby, 1950; Dubois, Doull and Coon, 1950; Cheng, 1951; Aldridge and Barnes, 1952). Rats pretreated with a suitable dose of β-diethylaminoethyl 3,3-diphenylpropylacetate survived doses of schradan that were fatal to normal animals (Davison, 1955).

Other drugs will inhibit the "non-specific" oxidase. For example, Fouts and Brodie (1956) and O'Brien and Davison (1958) showed that the potentiation of barbiturates by iproniazid reported by Allmark (1953), by Goldin, Dennis, Venditti, and Humphreys (1955), and by Sturtevant (1956), was due to the reduction in the liver oxidase activity. Isoniazid has also been reported by some of these workers to potentiate the effects of barbiturates. In view of the structural resemblance between isoniazid and iproniazid, it is possible that both inhibit the liver enzyme in the same way.

The activity of agents inhibiting this enzyme system has been investigated pharmacologically. The methods were based on measurement of the effects of drugs known to be substrates for this enzyme system, namely the hypnotic effects of pentobarbitone, the hypothermic action of chlorpromazine, and the toxicity of schradan. Potentiation of pentobarbitone hypnosis and chlorpromazine hypothermia results from inhibition of enzymic inactivation, whereas reduction of schradan toxicity results from inhibition of enzymatic activation.

METHODS

Male mice (LAC greys) weighing 16 to 25 g. were used. Drugs were injected intraperitoneally, except pentobarbitone which was given intravenously in a volume of 0.1 ml./10 g. of body weight. Room temperature was maintained at 22°. The doses of iproniazid, isoniazid, and β-diethylaminoethyl 3,3-diphenylpropylacetate used have no effect on the body temperature of the mice.

Pentobarbitone Sleeping Time.—This was the interval, in minutes, between giving 25 mg./kg. of pentobarbitone and the time at which the animals

were able to right themselves. The mean sleeping time of groups of 10 mice was determined.

Chlorpromazine Hypothermia.—Groups of mice were treated with 2.5 mg./kg. of chlorpromazine. The body temperature was measured 1 hr. later by a method previously described (Lessin and Parkes, 1957b) and the mean for the group determined. The test drugs were given at various intervals before the chlorpromazine.

Schradan Toxicity.—A graded response method was used. The mean survival time of groups of mice was determined after doses of from 25 to 50 mg./kg. of this compound. The interval between injection and death was called the survival time.

Results

Pentobarbitone Potentiation.—The potentiation of pentobarbitone sleeping time in mice given iproniazid (30 mg./kg.), isoniazid (30 mg./kg.) or β-diethylaminoethyl 3,3-diphenylpropylacetate (5 mg./kg.) 5 min., 1 hr., 2 hr. and 4 hr. before the pentobarbitone, is shown in Table I. Iproniazid and isoniazid exert their greatest potentiating action in 5 min., whereas the effect of β-diethylaminoethyl 3,3-diphenylpropylacetate appeared more slowly.

TABLE I

POTENTIATION BY IPRONIAZID, ISONIAZID, AND SKF 525A OF (*a*) PENTOBARBITONE SLEEPING TIME AND (*b*) CHLORPROMAZINE HYPOTHERMIA

Means ±S.E. of groups of 10 mice. (A) Sleeping time in min. after 25 mg./kg., i.v.; (B) body temp. 1 hr. after 2·5 mg./kg., i.p.

Potentiating Agent Given Before	Iproniazid (30 mg./kg.)	Isoniazid (30 mg./kg.)	SKF 525A (5 mg./kg.)
A. Pentobarbitone			
5 min. ..	17·8±1·1	16·3±1·4	12·0±1·3
1 hr.	18·7±1·1	17·0±1·1	22·4±1·6
2 ,, ...	15·7±1·3	13·6±1·4	22·6±1·6
4 ,, ...	9·7±0·6	12·0±1·3	21·2±1·3
No potentiator	7·5±0·5		7·1±1·0
B. Chlorpromazine			
5 min. ..	33·9±0·8	34·0±0·8	35·0±0·8
1 hr.	33·5±0·4	34·1±0·4	32·4±0·6
2 ,, ...	32·9±0·3	34·4±0·7	32·7±0·3
4 ,, ...	34·2±0·7	34·8±0·6	34·9±0·2
No potentiator	36·2±0·8	36·2±0·5	
,, treatment	37·4±0·06	37·7±0·2 (5 mice)	

TABLE II

EFFECT ON SURVIVAL TIME OF IPRONIAZID, ISONIAZID, AND SKF 525A GIVEN BEFORE SCHRADAN (50 MG./KG.)

Mean survival time±S.E. in min. (No. of mice used in parentheses)

Schradan Given After	Iproniazid (30 mg./kg.)	Isoniazid (30 mg./kg.)	SKF 525A (5 mg./kg.)
5 min. ..	21·1±1·0 (8)	23·6±1·8 (8)	16·2±0·4 (5)
30 ,, ..	20·2±0·9 (8)	22·9±0·9 (8)	19·9±0·8 (7)
1 hr. ..	19·0±0·9 (8)	21·7±0·7 (8)	22·2±0·9 (8)
4 ,, ..	18·7±1·3 (8)	21·7±1·3 (8)	20·1±1·3 (5)
24 ,, ..	14·9±0·6 (8)	14·1±0·7 (8)	
No inhibitor	14·8±0·4 (7)	14·9±0·7 (8)	13·9±0·06 (9)

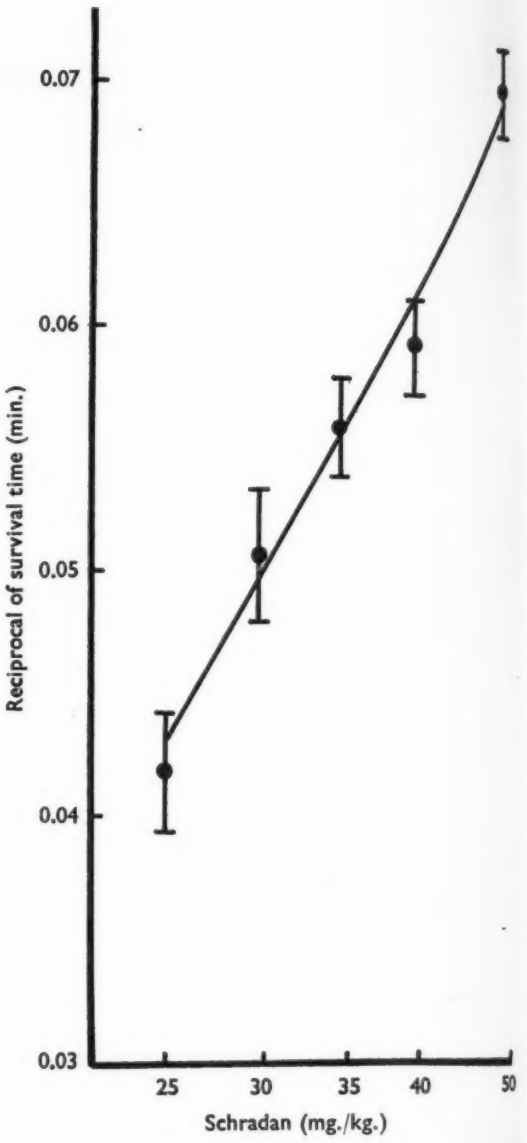

FIG. 1.—Relationship of schradan dose to survival time. n=55, r=0.80, b=0.091±0.019 (95% limits).

Chlorpromazine Hypothermia. — Chlorpromazine (2.5 mg./kg.) produced a fall in body temperature of 1.2 to 1.5° whereas in the pretreated animals the fall was 4.5 to 5.0°. As with pentobarbitone sleeping time, the first two compounds acted almost immediately, whereas the third took longer to exert its full effect (Table I).

Schradan Survival Time.—The doses ranged from 25 to 50 mg./kg.: below 25 mg./kg. animals did not die and above 50 mg./kg. there was little further shortening of the survival time (Fig. 1).

When β-diethylaminoethyl 3,3-diphenylpropylacetate (5 mg./kg.) was given to groups of mice at various intervals before 50 mg./kg. of schradan, the inhibitor exerted maximum protection when this interval was 1 to 2 hr. (Fig. 2). Iproniazid (30 mg./kg.) and isoniazid (30 mg./kg.) protected the animals from the toxic effects of schradan, although, as in the previous tests, the two hydrazides exert their full effect within a few minutes (Table II).

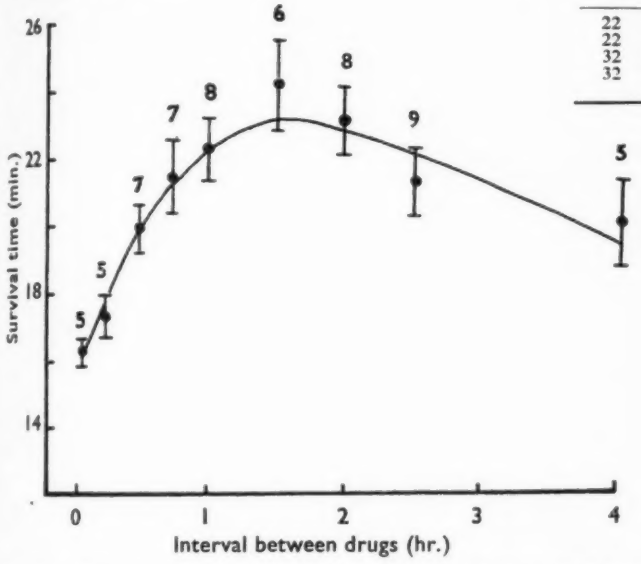

FIG. 2.—Effect on survival time of varying the interval between SKF 525A (5 mg./kg.) and schradan (50 mg./kg.). Range marks indicate S.E. and numerals the number of mice. 9 untreated mice survived 13.5 to 14 min.

TABLE III

EFFECT OF CHLORPROMAZINE AND RESERPINE UPON THE SURVIVAL TIME OF MICE TREATED WITH SCHRADAN (50 MG./KG.)

5 mice in each group. Effect of both drugs at 22° significant ($p < 0.01$).

Room Temp.	Drug	Dose (mg./kg.)	Interval between Drug and Schradan	Mean Survival Time ± S.E. (min.)
22	Chlorpromazine	10	1 hr.	All >30
22	—	—	—	14·5 ± 0·8
32	Chlorpromazine	10	1 hr.	18·7 ± 0·7
32	—	—	—	17·2 ± 1·2
22	Reserpine	2	4 hr.	21·9 ± 1·4
22	—	—	—	15·3 ± 0·7
32	Reserpine	2	4 hr.	14·0 ± 0·3
32	—	—	—	14·6 ± 0·6

To determine the effect of chlorpromazine on schradan toxicity, the animals were given 10 mg./kg. of chlorpromazine 1 hr. before schradan. When the experiment was carried out at an environmental temperature of 22°, chlorpromazine exerted a marked protecting action (Table III). On the other hand, when the environmental temperature was raised to 32° chlorpromazine no longer had any effect. Reserpine (2 mg./kg.) gave similar results, and afforded protection when the experiment was carried out at 22° but not at 32°.

In the last series of experiments various doses of iproniazid, isoniazid and β - diethylaminoethyl 3,3 - diphenyl - propylacetate were given at the appropriate interval before a fixed dose of schradan (50 mg./kg.), The dose/response curves for the three inhibitors are contained in Figs. 3, 4, and 5 respectively. The curves for iproniazid and isoniazid are similar and do not differ significantly from each other, but β-diethylaminoethyl 3,3-diphenylpropylacetate was clearly much more active than the other two.

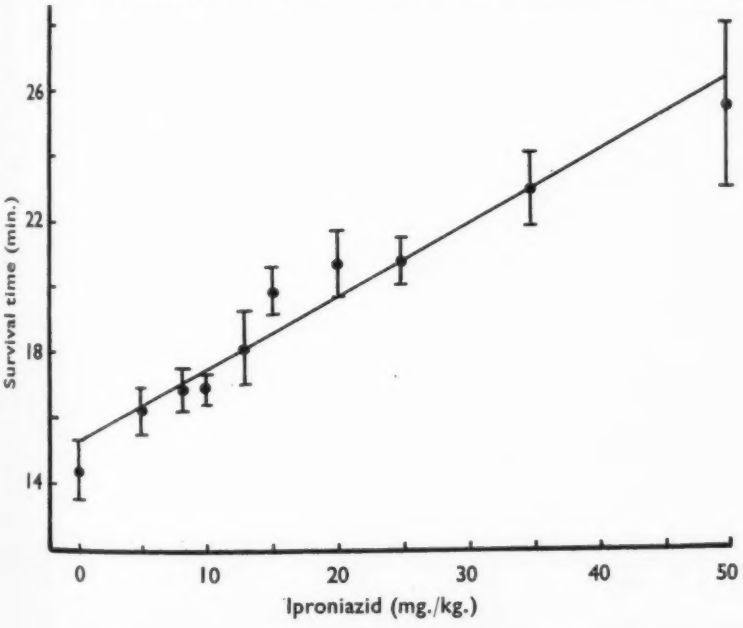

FIG. 3.—Effect of varying the dose of iproniazid upon survival time of mice treated with schradan (50 mg./kg.). Iproniazid 10 min. before schradan. n=83, r=0.71, b=0.22±0.015 (95% limits).

DISCUSSION

The results show that in mice iproniazid, isoniazid and β-di-

ethylaminoethyl 3,3-diphenylpropylacetate potentiated the pentobarbitone sleeping times and the hypothermic effects of chlorpromazine, and antagonized the toxic effects of schradan. In all three tests, iproniazid and isoniazid exerted their maximum potentiating effects very rapidly, whilst the peak effect with β-diethylaminoethyl 3,3-diphenylpropylacetate occurred about 1 to 2 hr. after its administration.

In all three tests β-diethylaminoethyl 3,3-diphenylpropylacetate was about 10 times more active than iproniazid or isoniazid.

The comparable results obtained for iproniazid and β-diethylaminoethyl 3,3-diphenylpropylacetate supported the view that potentiation of the pentobarbitone sleeping time and of the chlorpromazine hypothermia and the antagonism of schradan toxicity by these drugs was due to their known ability to inhibit the "non-specific" oxidase system. The results obtained with isoniazid resemble those with iproniazid, and it is reasonable to conclude that isoniazid is also an inhibitor of this enzyme system.

The toxicity of schradan is due almost entirely, as far as is known, to its metabolite, the phosphordiamide-*N*-oxide (Casida, Allen, and Stahmann, 1954). The production of this metabolite depends upon the integrity of the converting system. Chlorpromazine and reserpine have been shown to influence this system, when administered in circumstances allowing a fall in body temperature. On the other hand these two agents were ineffective when the temperature of the animals was not allowed to fall. Fuhrman (1947) showed that a lowered body temperature enhanced the hypnotic effects of pentobarbitone and he attributed this to the effect of lowered temperature upon the detoxicating enzyme.

Like that of many other enzyme systems, the activity of the liver oxidase system is therefore sensitive to changes in temperature, and caution is therefore indicated when evaluating inhibitors of this system by the pharmacological techniques described. The method based on schradan survival time is probably the most satisfactory to use once the effect of a fall in body temperature has been excluded.

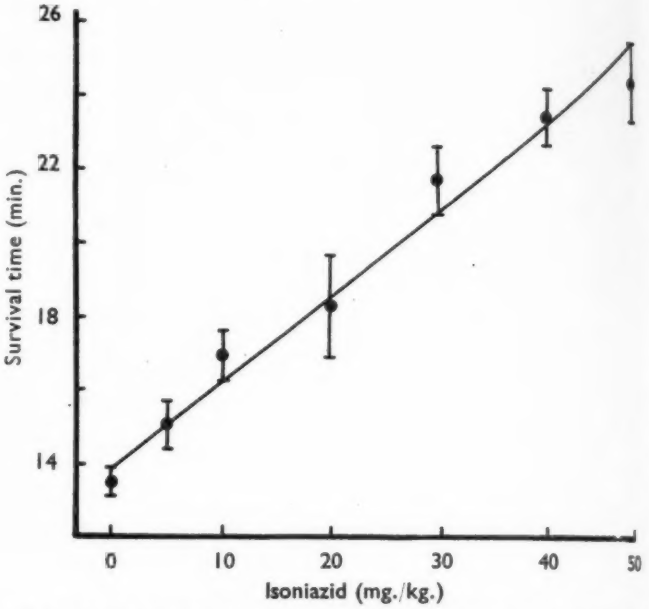

FIG. 4.—Effect of varying the dose of isoniazid upon survival time of mice treated with schradan (50 mg./kg.). Isoniazid 10 min. before schradan. n=57, r=0.88, b=0.23±0.01 (95% limits).

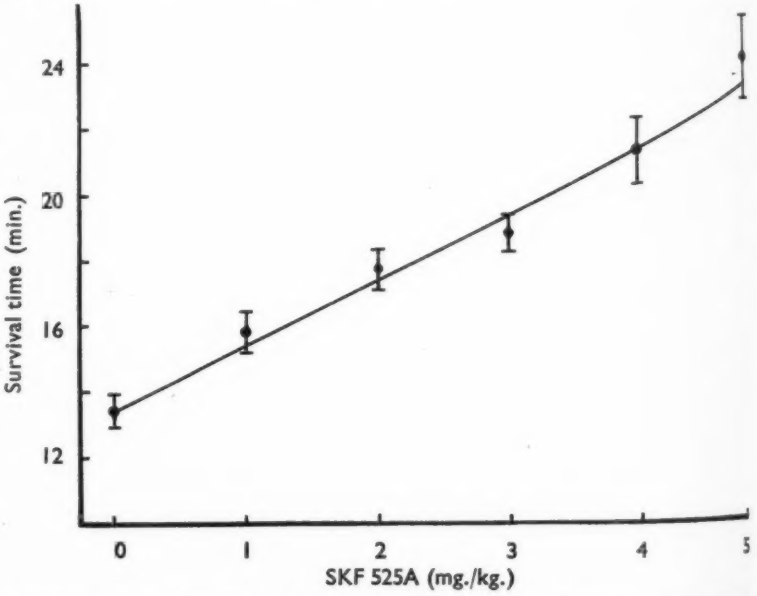

FIG. 5.—Effect of varying the dose of SKF 525A upon survival time of mice treated with schradan (50 mg./kg.). SKF 525A 1 hr. before schradan. n=61, r=0.76, b=1.98±0.14 (95% limits).

The author would like to thank Smith, Kline and French Laboratories, Philadelphia, for a generous gift of β-diethylaminoethyl 3,3-diphenylpropylacetate (SKF 525A).

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SPECIFICITY OF AMINE OXIDASE FOR OPTICALLY ACTIVE SUBSTRATES AND INHIBITORS

BY

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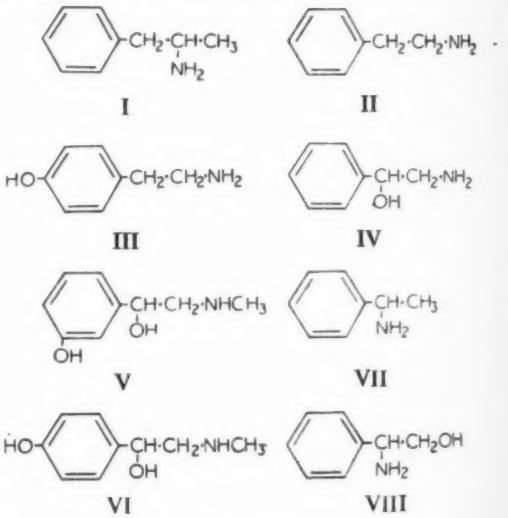
(RECEIVED APRIL 3, 1959)

A number of optically active amines have been tested as substrates or inhibitors of amine oxidase of rabbit and guinea-pig liver. The two stereoisomers of β-hydroxyphenethylamine were oxidized at the same rate by rabbit liver, but the guinea-pig liver extracts oxidized the D form more rapidly than the L form. The two stereoisomers of amphetamine were equally active as inhibitors of the rabbit liver oxidase, but with guinea-pig liver extracts dexamphetamine, the (+) form, was more potent as an inhibitor. In both species, 2-hydroxy-1-phenylethylamine was a weaker inhibitor than 1-phenylethylamine; in the rabbit liver (+) forms of these two amines were more potent as inhibitors.

The enzyme amine oxidase, which takes part in the biological inactivation of 5-hydroxytryptamine and many sympathomimetic amines, acts on compounds in which an amino group is attached to a terminal carbon atom. Compounds like amphetamine (α-methylphenethylamine) (I) and its derivatives, substances in which the amino group is not attached to a terminal carbon atom, are not oxidized; they act as competitive inhibitors of the enzyme (see Blaschko, 1952).

Mann and Quastel (1940) suggested that the central stimulating action of amphetamine might be due to its activity as an inhibitor of amine oxidase. This suggestion found some support in the observation that substances like amphetamine, in which the carbon atom adjacent to the benzene ring was not substituted, were stronger central stimulants, and at the same time stronger inhibitors of amine oxidase, than compounds like ephedrine, which carry a hydroxyl group in this position (Blaschko, 1940).

In the present paper the substrate and inhibitor specificities of a number of amines for amine oxidase have been studied. Attention was given to the question whether the steric configuration of amines with an asymmetric carbon atom had an influence upon their affinity for the enzyme. The substrates used were phenethylamine (II), tyramine (*p*-hydroxyphenethylamine) (III) and β-hydroxyphenethylamine (IV); of the last compound the D and L forms were available; the steric configuration of these two forms was determined some time ago (Pratesi and Grassi, 1953). A few experiments with both β*m*- and β*p*-dihydroxy-*N*-methylphenethylamine (V and VI respectively) are also reported. Phenylephrine, the (−) form of compound V, and the (+) form were available; we also used oxedrine (Sympatol), the (±) form of compound VI, as well as the (+) form; the latter compound is now known to have the L configuration (Pratesi, La Manna, Campiglio and Ghislandi, 1958).



The inhibitors tested were: amphetamine (I), 1-phenylethylamine (VII) and 2-hydroxy-1-phenylethylamine (VIII). Of all three compounds the two enantiomorphs were available.

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Material and Methods

The inhibitors used in this study were prepared by Dr. A. La Manna, of the Institute of Pharmaceutical Chemistry at the University of Pavia. All these substances were available as the hydrochlorides. In the following, the prefixes D and L refer to the steric configuration of the compounds; the signs (+) and (−) refer to the optical rotation of the hydrochlorides in aqueous solution.

Most experiments were carried out using a dialysed preparation of freshly ground rabbit or guinea-pig liver. Cat liver has also been used in a few experiments. The liver extracts were prepared in 0.067 M-sodium phosphate buffer of *p*H 7.4, as described by Barlow, Blaschko, Himms, and Trendelenburg (1955). The enzymatic activity was measured manometrically. The manometer flasks contained a fluid volume of 2.0 ml., plus 0.3 ml. of N-KOH. The temperature was 37.5° and the gas phase O_2. Unless stated otherwise, an inhibitor was tipped in from the side bulb, together with the substrate, at the zero time of the experiment. The initial substrate concentration, after tipping, was always 10^{-2} M.

Results

Substrates.—In rabbit liver preparations, the two stereoisomers of β-hydroxyphenethylamine were oxidized at the same rate. In one experiment, the oxygen uptake in the first 20 min. was 32 μl. O_2 for both the D and the L form, whereas with oxedrine, the (±) form of compound VI, the uptake was 18 μl. O_2. Oxedrine was oxidized at the same rate as the (+) form of compound VI.

In the guinea-pig liver extracts, there were two differences: first, oxedrine was always oxidized more rapidly than the two isomers of β-hydroxyphenethylamine, and, secondly, the L form of hydroxyphenethylamine was oxidized more slowly than the D form. In one of these experiments, the oxygen uptakes in the first 20 min. were: with oxedrine 33 μl., with L-β-hydroxyphenethylamine 10 μl. and with D-β-hydroxyphenethylamine 25 μl. There were no differences in the rates of oxidation of *βm*- and *βp*-dihydroxy-*N*-methylphenethylamine by guinea-pig liver extract: oxedrine and its dextrorotatory isomer and phenylephrine and the corresponding laevorotatory form were oxidized at similar rates.

Inhibitors.—In the rabbit liver, the two stereoisomers of amphetamine were equally active as inhibitors. This is illustrated by the following experiments: in one experiment with oxedrine as substrate the inhibitions during the first 15 min. were 91% with both dexamphetamine, the (+) isomer, and (−)-amphetamine in 10^{-2} M concentration; in another experiment the inhibitions were: with 3.33×10^{-3} M dexamphetamine 79%, and with 3.33×10^{-3} M (−)-amphetamine 71%, with 1.67×10^{-3} M dexamphetamine 57% and 1.67×10^{-3} M (−)-amphetamine 66%. These differences are well within the range of experimental error.

The action of the amine oxidase of the rabbit liver on tyramine was less strongly inhibited than that on oxedrine, indicating that tyramine had a higher affinity for the enzyme than oxedrine. In one experiment in which 10^{-2} M dexamphetamine was the inhibitor the inhibition with tyramine as substrate was 78%; with 10^{-2} M (−)-amphetamine as inhibitor it was 80%. In another experiment, the inhibition of the oxidation of tyramine by 10^{-2} M dexamphetamine was 74%; with phenethylamine as substrate the inhibition was 33%. In the rabbit the rate of oxidation of phenethylamine exceeded that of tyramine (see also Alles and Heegaard, 1943); the experiment just described shows that the liver enzyme of this species also has a higher affinity for phenethylamine than for tyramine.

In the guinea-pig liver preparations, tyramine was so much more rapidly oxidized than phenethylamine that it was difficult to compare the oxidation of these two amines in one and the same experiment, using the same amounts of enzyme. Different amounts of the guinea-pig liver extract had therefore to be used with the two substrates. For the oxidation of tyramine, 0.4 ml. of extract was chosen as a suitable amount in each flask; for the oxidation of phenethylamine, 1.6 ml. of the extract was used. Under these conditions, the oxygen uptakes were of a similar order, namely 59 μl. in 15 min. with tyramine and 67 μl. with phenethylamine. The inhibitions in the presence of 10^{-2} M-dexamphetamine were: 95% with tyramine as substrate and 43% with phenethylamine. In other words, although in the guinea-pig liver tyramine was much more rapidly oxidized than phenethylamine (see Blaschko, Richter and Schlossmann, 1937a; Randall, 1946), the enzyme had a higher affinity for phenethylamine than for tyramine.

A comparison of the inhibitory action of the two enantiomorphs of amphetamine revealed a difference between the guinea-pig and the rabbit oxidases. With rabbit liver, there were no marked differences in the inhibitory action of the two isomers, but with guinea-pig liver the dextrorotatory form was always a stronger inhibitor. This difference was noted not only with the two samples of the amphetamine hydrochlorides prepared at Pavia, but it was also present using dexamphetamine (Dexedrine) sulphate and (−)-amphetamine (Levedrine) sulphate, kindly

TABLE I

OXIDATION OF TYRAMINE (10^{-2} M) BY RABBIT AND GUINEA-PIG LIVER PREPARATIONS AND INHIBITION BY THE TWO STEREOISOMERS OF AMPHETAMINE (10^{-2} M)

The values give the oxygen uptakes in μl. during the first 15 min.

	Tyramine Alone	With Dexamphetamine	With (−)-Amphetamine
Rabbit liver ..	66	14·5	13
Guinea-pig liver	71	4·5	17

given by Messrs. Smith, Kline and French. Table I shows the results with two of these experiments, in which 10^{-2} M-tyramine was the substrate.

Amphetamine (I) differs from the other two inhibitors studied, 1-phenylethylamine (VII) and 2-hydroxy-1-phenylethylamine (VIII), in that in amphetamine the amino group is not attached to the carbon atom that carries the phenyl group, whereas in 1-phenylethylamine and 2-hydroxy-1-phenylethylamine both groups are attached to the same carbon atom.

1-Phenylethylamine was found to be a moderately strong inhibitor of amine oxidase. In the rabbit liver extracts (+)-1-phenylethylamine was a more active inhibitor of the enzyme than dexamphetamine. On the other hand, the (+)-2-hydroxy-1-phenylethylamine was a weaker inhibitor than dexamphetamine. Similarly, in two experiments with cat liver preparations, it was found that (+)-1-phenylethylamine was a stronger inhibitor than dexamphetamine.

In the guinea-pig liver extracts dexamphetamine was a stronger inhibitor than either (+)-1-phenylethylamine or (+)-2-hydroxy-1-phenylethylamine. These differences between the rabbit and the guinea-pig liver enzymes are shown in Figs. 1 and 2, taken from experiments in which 10^{-2} M-tyramine served as substrate. It can be seen that the rate of oxidation of tyramine by rabbit liver was most strongly inhibited by 1-phenylethylamine; then comes amphetamine and lastly 2-hydroxy-1-

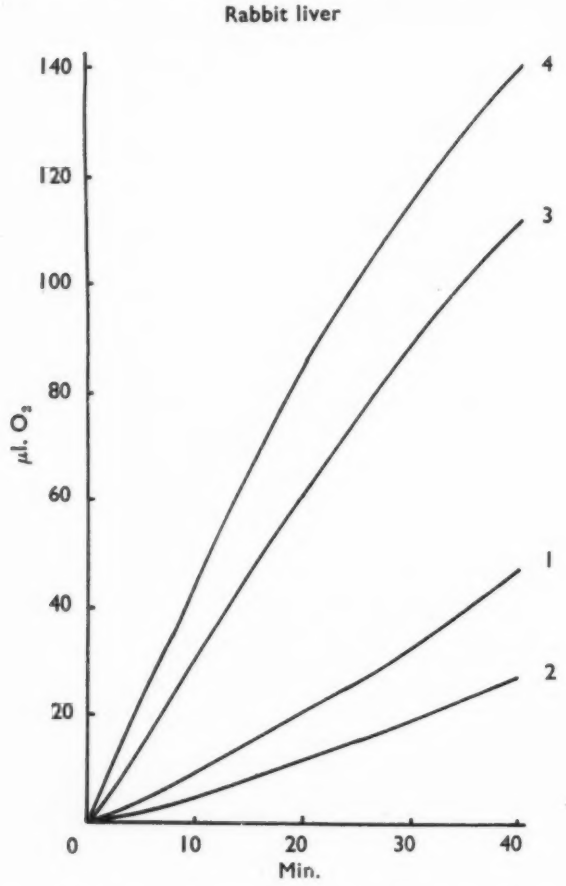

FIG. 1.—Inhibition of amine oxidase of rabbit liver by: (1), dexamphetamine; (2), (+)-1-phenylethylamine; (3), (+)-2-hydroxy-1-phenylethylamine, and (4), tyramine alone. The tyramine concentration and that of the inhibitors was 10^{-2} M.

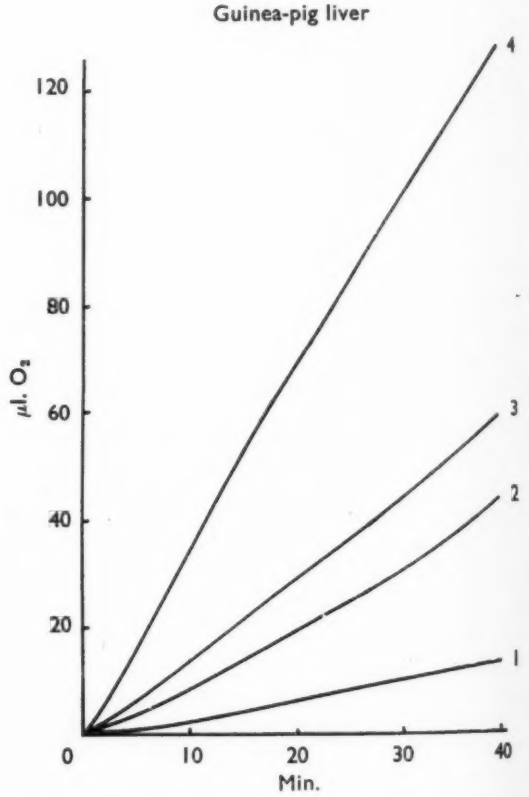

FIG. 2.—Inhibition of amine oxidase of guinea-pig liver by: (1), dexamphetamine; (2), (+)-1-phenylethylamine; (3), (+)-2-hydroxy-1-phenylethylamine, and (4), tyramine alone. The tyramine concentration and that of the inhibitors was 10^{-2} M.

phenylethylamine (Fig. 1); with guinea-pig liver the oxidation was most strongly inhibited by amphetamine (Fig. 2).

The two enantiomorphs of both 1-phenylethylamine and 2-hydroxy-1-phenylethylamine were tested. In the experiments with guinea-pig liver, no difference was noted between the inhibitory activity of the two enantiomorphs, but with rabbit liver the dextrorotatory forms were more active as inhibitors. This was tested in a series of experiments in which 10^{-2} M-oxedrine was used as substrate and an inhibitor concentration of 10^{-3} M. In three such experiments with 1-phenylethylamine the inhibitions in the first 15 min. were: with (−)-1-phenylethylamine, 34, 33 and 12%; and with (+)-1-phenylethylamine, 81, 88 and 62%. In four similar experiments with 2-hydroxy-1-phenylethylamine, the corresponding values were: with (−)-2-hydroxy-1-phenylethylamine, 17, 34, 56, and 52%; and with (+)-2-hydroxy-1-phenylethylamine, 52, 79, 69, and 71%.

Discussion

Many observations have been made on the substrate specificity of amine oxidase, and the observation reported in this paper, that there exist species differences in the pattern of substrate and inhibitor specificities, finds many parallels in earlier work. Little, however, was known of the stereochemistry of the oxidase. In the present work we have been able to test several substances with an asymmetric carbon atom, and of each of these both enantiomorphs were available.

As a result of this study, it can be said that the presence of a centre of asymmetry may confer different affinities (or rates of oxidation) on two enantiomorphs, and also that this difference between two enantiomorphs depends on the source of enzyme. In extracts from rabbit liver, the D and L isomers of β-hydroxyphenethylamine were oxidized at similar rates, but in extracts from guinea-pig liver the D form was more rapidly oxidized than the L form. This observation is of interest in connexion with old observations on adrenaline (Blaschko, Richter, and Schlossmann, 1937b), in which it was found that the initial rate of oxidation of (−)-adrenaline by guinea-pig liver enzyme was faster than that of (+)-adrenaline. That (−)-adrenaline has the D configuration has now been established by a stereochemically unequivocal route (Pratesi, La Manna, Campiglio, and Ghislandi, 1958). Adrenaline, as a catechol derivative, is not a very convenient substrate for manometric experimentation, as oxygen uptake not catalysed by amine oxidase may vitiate results. It is of interest, therefore, that a similar relationship between D and L isomers has now been established for compounds which do not carry a phenolic hydroxyl group.

Another species difference noted was in the relative rates of oxidation of different substrates of the oxidase. In the rabbit liver oxedrine was less readily oxidized than β-hydroxyphenethylamine, but in the guinea-pig liver this order was reversed. The experiments also confirmed the enormous differences in the rate of enzymatic oxidation of phenethylamine, which was rapidly oxidized by the rabbit liver, but slowly by the guinea-pig liver; the experiments with inhibitors suggest that the affinity of the guinea-pig liver enzyme for phenethylamine is high although its rate of oxidation is low.

The well-established general rule, that compounds in which the amino group is not attached to a terminal carbon atom are inhibitors and not substrates of amine oxidase, was confirmed for 1-phenylethylamine and 2-hydroxy-1-phenylethylamine. In the series studied, the introduction of a hydroxyl group in the 2-position relative to the amino group lowers affinity for amine oxidase; this is an analogy in the 1-phenylethylamine series for what is already known in the much more extensively studied group of derivatives of phenethylamine.

A species difference was also found in the comparison of the inhibitory actions of dexamphetamine and (+)-1-phenylethylamine. In the rabbit liver, the latter was the stronger inhibitor (see Fig. 1), but in the guinea-pig liver dexamphetamine inhibited more strongly (see Fig. 2).

A difference was also seen in the inhibitory action of the three inhibitors studied. In the rabbit liver, no significant difference in the inhibitory potency of the two stereoisomers of amphetamine was noted, but both (+)-1-phenylethylamine and (+)-2-hydroxy-1-phenylethylamine were more active as inhibitors than the corresponding laevorotatory forms. Conversely, with guinea-pig liver there was no difference when the two forms of 1-phenylethylamine and 2-hydroxy-1-phenylethylamine were compared, but dexamphetamine was a stronger inhibitor than (−)-amphetamine.

These experiments show that the steric configuration of derivatives of phenylethylamine, of 1-phenylethylamine and of amphetamine may be a factor in determining the affinity for amine oxidase. Can these observations be related to the analeptic properties of the amphetamines? The dextrorotatory form of amphetamine is said to be

more active as an analeptic than the laevorotatory form (see Bovet and Bovet-Nitti, 1948). This is the same difference as that established for the inhibition of the guinea-pig liver enzyme. However, no such difference was found for the rabbit liver enzyme. Moreover, the lack of parallelism between anti-amine-oxidase activity and analeptic action is even more apparent when amphetamine and 1-phenylethylamine are compared. The latter compound was found to be a good inhibitor of amine oxidase, in the rabbit liver even stronger than amphetamine. However, the compound is without the strong central action of amphetamine. This aspect is at present being studied by Drs. E. Grana and L. Lilla at Pavia: they have found that both enantiomorphs of 1-phenylethylamine have a much weaker analeptic action than dexamphetamine in the rat although the action of (+)-1-phenylethylamine on amine oxidase in this species is very similar to that of dexamphetamine. This work will be separately reported. It is difficult to see how these results can be reconciled with the idea that inhibition of amine oxidase can account for the analeptic action of amphetamine and related compounds.

The authors are grateful to Professor K. Zeile for the two isomers of *βm*-dihydroxy-*N*-methylphenethylamine used in this study, and Miss Rosemary Hawes for technical assistance. This work was carried out during the tenure by one of us (P. P.) of a grant from the Fondazione Guido Donegani, awarded by the Italian Accademia Nazionale dei Lincei.

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THIOPENTONE AND BUTHALITONE: THE RELATIONSHIP BETWEEN DEPTH OF ANAESTHESIA, PLASMA CONCENTRATION AND PLASMA PROTEIN BINDING

BY

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For 24 hr. after intravenous administration of buthalitone or thiopentone, plasma concentrations in young human subjects have been followed. Buthalitone was distributed to the tissues more rapidly but was metabolized at a slower rate than thiopentone. The relationships between these findings and differences in plasma protein binding and oil/water partition coefficients were studied. It is suggested that some of the differences observed in potency between the substances is a reflection of differences in their modes of distribution. No relationship was found between speed of recovery from anaesthesia and plasma barbiturate concentrations.

Of the new ultra-short-acting thiobarbiturates, the most commonly employed is buthalitone sodium, the sodium salt of 5-allyl-5-isobutyl-thiobarbituric acid. It was introduced as an anaesthetic by Weese and Koss (1954) and has achieved its greatest popularity on the European continent. In Britain some authors regard it as useful for anaesthesia of brief duration (Young, 1956; Henderson and Mackett, 1957), while others conclude that it offers no advantage over thiopentone sodium, the sodium salt of 5-ethyl-5-(1-methylbutyl)-thiobarbituric acid (Simmons and Blanshard, 1957; O'Mullane, 1957). It is generally agreed that buthalitone appears to be a less potent anaesthetic than thiopentone when given in the same dose. The present investigation had three main objects: first, to compare the rates of elimination of the two substances from the blood stream after intravenous injection and thereby to deduce, if possible, any difference there might be in the rates of metabolism of the two; second, to attempt to relate changes in plasma concentrations with the clinical signs of recovery from anaesthesia; and third, to compare two physical attributes, the plasma protein binding and the oil/water partition coefficients, in an attempt to account for the differences in clinical activity of the two drugs.

METHODS

Clinical.—The patients were otherwise healthy male subjects, aged 18 to 37 years, weighing 58.5 to 86.2 kg., who underwent minor operative procedures such as incisions, manipulations, and excisions of cysts. Premedication consisted solely of atropine sulphate (0.6 mg. given subcutaneously) 45 min. before operation. Doses both of buthalitone (16 patients) and of thiopentone (10 patients), based on 11 mg./kg. body weight, were administered as 10 and 5% (w/v) solutions respectively at a rate of 1 ml./sec. The drug was injected into a vein in the antecubital fossa, and blood samples were withdrawn from a vein in the opposite arm at −1, 2, 4, 8, 16, 32 min., and 1, 2, 4, 8, and 24 hr. from the mean time of the injection. In most cases some supplementary anaesthesia was required, and nitrous oxide, oxygen, and trichlorethylene or cyclopropane and oxygen were administered. In a few cases muscle relaxants, with or without controlled respiration, were needed. Three patients were given a second dose of buthalitone 16 min. after the first. Three tests were applied to six subjects who received buthalitone but no supplementary anaesthesia whenever blood samples were taken. The observations were recorded by a witness otherwise independent of the investigation, and consisted of: (1) Response to pin-prick. A movement resulting from two or more out of three such stimuli applied to the back of the hand was recorded as being a positive response. (2) Response to simple commands such as "Put out your tongue" and "Lift up your right hand." Obedience to any single such command was recorded as positive. (3) The ability of the patient to walk unaided.

Laboratory.—Blood was transferred to tubes containing potassium oxalate (1 mg./ml.) and centrifuged within 1 hr. of collection. Thiobarbiturate concentrations were estimated by the method of Crawford and Kane (1956) which involved extraction

of the substance into chloroform. Two ml. of plasma was used for each estimation. To establish the suitability of the method for the estimation of buthalitone, analyses were performed on plasma samples, taken from patients receiving no barbiturates, to which known amounts of buthalitone in the range 0 to 50 μg. had been added. A linear relationship was found between "corrected" absorbance (Crawford and Kane, 1956) and amount of buthalitone. The agreement between replicate estimates was of the order ±10%.

Brodie, Mark, Papper, Lief, Bernstein, and Rovenstine (1950) have found that methods of barbiturate estimation involving extraction into a highly polar solvent such as chloroform may give results which are falsely high if plasma concentrations are followed for prolonged periods. This is due to the extraction of a metabolic product, a pharmacologically inactive carboxylic acid derivative, which has a similar ultra-violet absorption spectrum. They therefore used a method involving extraction into light petroleum in order to obtain "true" thiopentone concentrations. To establish whether the chloroform-extraction method estimates the barbiturate alone or in addition its metabolic product, a number of 4 hr. plasma samples were estimated in duplicate, employing the method of Brodie *et al.* (1950) and the present method. In addition, one 4-hr. sample was estimated by infra-red as well as ultra-violet spectrophotometry with the same object in view, since the infra-red spectrum of a metabolite is very unlikely to be the same as that of the drug. Twenty ml. of plasma was extracted with 250 ml. chloroform. The chloroform phase was filtered through a No. 1 Whatman filter and extracted with 50 ml. of 0.1 N-NaOH. The aqueous phase was acidified with HCl and re-extracted with 4 × 10 ml. of chloroform. The chloroform extracts were dried with Na_2SO_4 and evaporated on to 300 mg. KBr. A pressed disc was made (Stimpson and O'Donnell, 1952) and the infra-red spectrum was obtained on a Perkin-Elmer model 21 spectrophotometer over the range 6 to 10 μ. The quantity of buthalitone had been found from trial experiments to be proportional to the "corrected" absorbances at 6.55 and 8.65 μ. An attempt to develop an infra-red method for thiopentone failed owing to the lack of strong bands for this compound in regions of the infra-red spectrum in which interference from extracted constituents of plasma was not encountered. The results of these preliminary experiments (Table I) confirmed that for thiopentone the concentrations recorded by the method of Crawford and Kane (1956) were probably too high. For buthalitone, however, the different methods showed good agreement, indicating a probable lack of interference from metabolic products.

TABLE I

COMPARISON OF METHODS OF ESTIMATING BARBITURATES

Duplicate results on 5 separate 4-hr. samples, expressed in μg./ml.

No.	Barbiturate	Ultra-violet		Infra-red
		Chloroform Extraction	Light Petroleum Extraction	Chloroform Extraction
1	Thiopentone	4·4	3·3	—
2	,,	7·1	4·1	—
3	Buthalitone	9·7	11·6	—
4	,,	0·8	0·6	—
5	,,	10·3	—	9·7

Plasma protein binding was estimated by dialysing fresh oxalated human plasma whose protein concentration was found to be 70 mg./ml. (albumen 46 mg./ml., globulin 24 mg./ml.) in ¼ inch "Visking" tubing bags against solutions of the barbiturate. These solutions were made up in M/15 phosphate buffer of *p*H 7.4 with olive oil at 37° for 3 hr., and 37° for 24 hr. Trial experiments revealed that binding increased during the first 24 hr., thereafter gradually decreasing, probably because of slow protein denaturation. Penicillin 100 units/ml. and streptomycin 100 μg./ml., which were found not to interfere with either the extent of binding or the protein or barbiturate estimations, were added to the barbiturate solutions in order to prevent the growth of bacteria during the incubation period. Because the plasma protein became diluted by 10 to 15% during the experiments, binding is expressed as the amount of barbiturate/mg. protein.

Oil/water partition coefficients were found by shaking the barbiturate solutions in phosphate buffer of *p*H 7.4 with olive oil at 37° for 3 hr., and estimating the amounts of barbiturate in the aqueous layer before and after shaking.

RESULTS

Blood Barbiturate Concentrations.—Fig. 1 shows the mean plasma concentrations after injections of buthalitone and thiopentone. Although the general trend of the plasma concentrations was similar in the two groups, there were two main differences. In the first place, in spite of the similarity of dose, the concentrations of thiopentone during the first 32 min. after a single dose were significantly higher than those of buthalitone ($P<0.01$). On the other hand, the concentration of thiopentone from 2 hr. onwards declined more rapidly and the difference in concentrations of the two substances again became significant at 8 ($P<0.05$) and 24 hr. ($P<0.001$).

Clinically, most of the six patients who received buthalitone but no supplementary anaesthesia were completely unresponsive to all stimuli at 2 min., and at 32 min. were able to walk unaided. Individually, however, their plasma concentrations varied only slightly during this period and no relation was found between recovery from anaesthesia and the plasma thiobarbiturate concentration. For example, one patient was

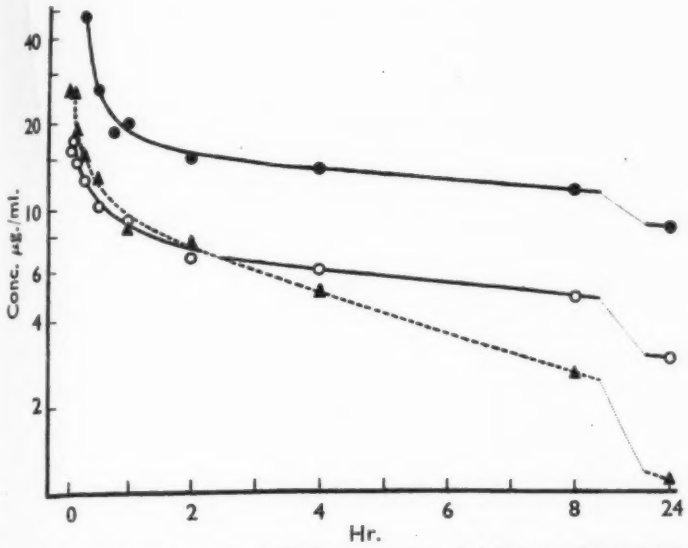

FIG. 1.—Mean plasma concentrations after intravenous administration of buthalitone 11 mg./kg. injected at 0 and 16 min. (n=3), ●———●; buthalitone 11 mg./kg. at 0 min. (n=16), O———O; thiopentone 11 mg./kg. at 0 min. (n=10), ▲- - -▲; standard errors are smaller than symbol used for mean except at 8 and 24 hr.

completely unconscious at 2 min. with a plasma concentration of 13.4 μg./ml., and at 4 min. he responded to pin-prick and obeyed simple commands, although he then had a plasma concentration of 13.9 μg./ml. Another subject was unconscious at 2 min. (10.0 μg./ml.), able to respond to pin-prick at 4 min. (12.3 μg./ml.), obeyed commands at 8 min. (13.8 μg./ml.) and at 32 min. was able to walk unaided, although his plasma concentration was then 10.1 μg./ml.

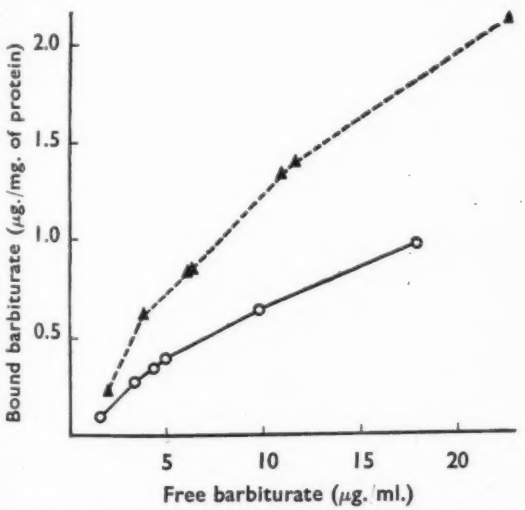

FIG. 2.—Barbiturate bound by plasma proteins. Thiopentone, ▲- - -▲; buthalitone, O———O.

The mean plasma concentrations found in the three subjects who received two doses of buthalitone are illustrated from 18 min. onwards in Fig. 1. The concentrations attained 2 min. after the second dose are more than twice as high as those after one dose, but from 4 hr. onwards the decline runs parallel to that following the single dose. Recovery of consciousness in these three patients was delayed until about 20 min. after the second dose, but again no relation was found between this recovery and the plasma thiobarbiturate level.

Plasma Protein Binding.—Dialysis experiments showed that thiopentone was bound to a much greater extent than was buthalitone, and that increasing barbiturate concentrations led to a relative fall-off in the degree of binding, as partial saturation of the protein occurred (Fig. 2).

Oil/Water Partition Coefficients.—The mean of three estimations of the coefficients for each substance indicated that thiopentone was relatively more soluble in fat than was buthalitone. The olive oil/water concentration ratios at *p*H 7.4 were 58:1 for thiopentone and 18:1 for buthalitone.

DISCUSSION

The present studies indicate that there are significant biochemical and physico-chemical differences between thiopentone and buthalitone. These differences become of general interest if the two are found to be related. Although Butler (1950) suggested that such relationships are valid only between drugs as closely related as those in a homologous series, it is felt that thiopentone and buthalitone resemble each other sufficiently closely to be able to draw certain conclusions from these investigations.

In the first place, the finding that, following intravenous administration, the initial concentrations of thiopentone were significantly higher than those of buthalitone indicated that the former substance left the circulation less rapidly than the latter. This is to be expected if, as was found, plasma protein binding was more extensive in the case of thiopentone, leaving less free barbiturate to be distributed to the tissues. The finding, that, from 2 hr. after injection onwards, the slope of thiopentone concentrations was considerably steeper than that of buthalitone,

indicated a more rapid rate of metabolism. That this slope does indicate a rate of metabolism is suggested by the parallelism of the slopes following the different doses, reported here for buthalitone, and by Brodie *et al.* (1950) for thiopentone. It is possible that differences in protein binding are manifest not only in the plasma but also in the liver, and that the rates of metabolism are influenced by binding to the enzymes concerned. However, other factors, such as chemical structure and polarity, probably influence the rate as well, and it is not therefore suggested that protein binding is the only one. As far as the nature of the metabolic pathway is concerned, thiopentone undergoes terminal oxidation of its 1-methyl-butyl side chain (Brodie *et al.*, 1950; Cooper and Brodie, 1957), but the changes which occur to buthalitone are not known. Our findings suggest that terminal oxidation is unlikely as we were unable to find evidence of a carboxylic acid derivative with the extraction procedures used.

As a central depressant agent, thiopentone is much more potent than buthalitone. Simmons and Blanshard (1957) and Orkin, Morales, Fujita, and Gabuya (1958) both reported a clinical potency ratio of about 2:1. Rivett (personal communication) found that, when administered intravenously to mice, the anaesthetic dose of buthalitone was three times that of thiopentone. O'Mullane (1957) showed that the same relative amounts were needed to produce equivalent electroencephalographic changes in human subjects. The question arises as to whether it is possible to account for any of this difference in potency. Mark, Burns, Brand, Campomanes, Trousof, Papper, and Brodie (1958) showed a remarkable correlation between oil/water partition coefficients of various barbiturates and the speed with which they enter the brain after intravenous injection, the ones with the highest coefficients entering most rapidly. On this basis, thiopentone probably enters the brain faster than buthalitone as it has a higher coefficient. There appears, therefore, to be a tendency for more thiopentone to enter the brain, but, because less is bound to plasma protein, more buthalitone appears to enter the tissues generally. It seems highly probable that this may account for some of the difference in potency between the two substances. In other words, potency may be partly a manifestation of physical distribution, and that one of the factors which makes thiopentone a more potent central depressant than buthalitone is simply that more reaches the brain in the first few minutes. However, other factors are involved, as shown by the recent findings of Parker and Aldridge (personal communication). They have assessed the inhibitory action of various barbiturates on oxidative phosphorylation of brain and liver mitochondria, and have shown that, in this respect, thiopentone is between two and three times as effective as buthalitone. While not proven, it is probably reasonable to assume a parallelism between such inhibition and central depressant action.

Wyke (1957) has pointed out that one implication of protein binding is that alterations in plasma protein levels may affect sensitivity of patients to administered barbiturates, and that subjects who are protein depleted or acidotic become more sensitive to their action, because a greater proportion of the administered dose remains unbound. However, Goldbaum and Smith (1954), who investigated binding by an ultra-filtration method and employed relatively low concentrations of protein for most of their experiments, suggest that variations in protein concentration or in *p*H found under clinical conditions are unlikely to influence to any significant extent the proportion of barbiturate remaining unbound.

Whether the physical attributes of these two substances determine their potency or not, it seems probable that they influence their mode and rate of distribution and metabolism and also their duration of action.

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ACTIONS OF SOME ANALOGUES OF 5-HYDROXYTRYPTAMINE ON THE ISOLATED RAT UTERUS AND THE RAT FUNDUS STRIP PREPARATIONS

BY

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The search for substances which antagonize 5-hydroxytryptamine, and for those which act like it, has been extended to cover 5-substituted indoles which are analogues of 5-hydroxytryptamine, rather than analogues of tryptamine like the compounds previously studied by us. The isolated rat uterus and rat fundus strip preparations have been used to determine activity. The relationships between structure and activity of the compounds studied were not the same on the two preparations nor were they the same from one homologous series to another. These differences may be partly explained by the presence of amine oxidase in the rat fundus, as Vane (1959) has suggested, and by supposing that it is absent from the rat uterus. None of the compounds had marked antagonist activity. The most active antagonist on the rat uterus was 3-(2-aminopropyl)-5-benzyloxyindole, but this was less potent than 5-benzyloxygramine. On the rat fundus strip, however, the only antagonist, 5-benzyloxy-3-(2-dimethylaminoethyl)indole, was more active than 5-benzyloxygramine. On both preparations the most active stimulant was 3-(2-aminopropyl)-5-hydroxyindole, which was about half as potent as 5-hydroxytryptamine. The next most active were 3-(2-aminopropyl)-5-methoxyindole and bufotenine.

A previous paper (Barlow and Khan, 1959) described our search among analogues of tryptamine for substances which act like tryptamine and/or 5-hydroxytryptamine and those which antagonize tryptamine and/or 5-hydroxytryptamine. We found that substitution of alkyl groups in the amino group of the side-chain of tryptamine produced an increase in tryptamine-like and 5-hydroxytryptamine-like activity.

A series of derivatives of 5-hydroxytryptamine which have alkyl substituents in the side-chain amino group was prepared, and the study also included: (1) the corresponding 5-benzyloxy compounds, which, from previous work (see, for example, Gaddum, Hameed, Hathway, and Stephens, 1955), might be expected to be antagonists of 5-hydroxytryptamine, (2) the effects both on stimulant and antagonistic activity of substituting a methyl group in the side-chain in the α position relative to the amino group, this substitution making the compound resistant to attack by amine oxidase, and (3) the actions of 5-methyl and/or 5-methoxy compounds. These, being non-phenolic, might indicate how much the phenolic nature of the 5-hydroxyl group determined the potency of 5-hydroxytryptamine.

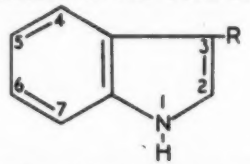
We have continued to use the isolated rat uterus and rat fundus strip preparations for measuring both agonistic and antagonistic potencies because they are convenient preparations which respond to tryptamine and 5-hydroxytryptamine. The compounds tested are shown in Table I. Although they are referred to as bases, a suitable salt was always used.

METHODS

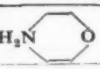
Compounds.—Analogues of 5-hydroxytryptamine have already been prepared by Speeter and Anthony (1954), Stoll, Troxler, Peyer and Hofmann (1955), Speeter (1955), and Young (1958). Samples of some of these have kindly been made available to us. The other compounds were prepared by the method of Speeter and Anthony (1954) starting from 5-benzyloxyindole. The intermediate 5-benzyloxy-3-(2-dialkylaminoethyl)indoles were de-benzylated by hydrogen and palladium-charcoal (10%) at atmospheric pressure. Methanol was used as solvent and the mixture warmed, if necessary. The quaternary salts of 5-benzyloxy-3-(2-dimethylaminoethyl)indole could not be de-benzylated catalytically:

TABLE I

STIMULANT ACTIVITY OF THE COMPOUNDS STUDIED



Microanalyses are by Dr. J. W. Minnis (Department of Biochemistry); melting points were taken on a Kofler hot stage. An asterisk refers to remarks in the section on drugs in the text. EtOH = ethanol, MeCOMe = acetone, EMK = ethyl methyl ketone, and Et_2O = ether. † Indicates that the dose/response curve was not parallel to that of 5-hydroxytryptamine. ‡ Indicates that these compounds have been prepared previously by other workers: No. 2 by Wieland, Konz, and Mittasch (1934), who recorded m.p. 210°: No. 7 by Speeter (1955), who recorded m.p. 213 to 214°. Compounds 4 and 11 have been prepared as bases and oxalates by Stoll, Troxler, Peyer, and Hofmann (1955). The explanation of (S), (C), (Cu), and (V) is given in the acknowledgments section and refers to the source of these compounds. Number of experiments are shown in parentheses after equipotent molar ratios. (I) Indicates that results with these compounds were recorded by Barlow and Khan (1959) and are included for purposes of comparison.

Comp. No.	Ring Sub-stituent	Side-chain (R) (Trivial Name)	Salt	Crystallized from	m.p. (°C.)	Found		Theory		Stimulant Activity: Equipotent Molar Ratios: Mean ± S.E.	
						C	H	C	H	Rat Uterus 30°	Rat Fundus Strip 37°
1	5-OH	$-CH_2CH_2NMe_2$ (Bufotenine)	Base Fumarate	$MeCOMe/Et_2O$ EtOH	125–6°* 228–30° (dec)	70·9 64·1	7·77 6·79	70·6 64·2	7·92 6·91	2·7 ± 1·6 (3)	3·1 ± 1·8 (3)
2	5-OH	$-CH_2-CH_2-N(CH_3)_2$	Methiodide	EtOH	210–11°‡					>20,000 (2)	>10,000 (2)
3	5-OH	$-CH_2-CH_2-N(CH_3)_2$	Ethiodide	EtOH	192–3°	46·6	5·61	46·6	5·89	>20,000 (2)	>10,000 (2)
4	5-OH	$-CH_2-CH_2-N(C_2H_5)_2$ (*N'N'*-Diethyl-5-hydroxytryptamine)	HCl	EtOH/EMK	169·5–170°	62·9	7·55	62·5	7·90	9·0 ± 2·6 (3)	18 ± 9·5 (3)
5	5-OH	$-CH_2CH_2N(C_3H_7)_2$ (*N'N'*-Dipropyl-5-hydroxytryptamine)	HCl	EtOH/EMK	204–5°	64·7	8·33	64·8	8·52	39 ± 1·5 (3)	16 ± 4·0 (6)
6	5-OH	$-CH_2-CH_2-N(isoC_3H_7)_2$	HCl	$EtOH/EMK/Et_2O$	109–11°	64·5	8·59	64·8	8·52	16 ± 0 (3)	4·5 ± 1·5 (3)
7	5-OH	$-CH_2-CH_2-N(C_4H_9)_2$	HCl	EtOH/EMK	212–15°‡	66·9	8·97	66·5	9·02	1,111† (2)	151 ± 40 (3)
8	$5-OC_7H_7$	$-CH_2-CH_2-N(CH_3)_2$	HCl	(I)						>20,000 (2)	625 ± 135 (4)
9	$5-OC_7H_7$	$-CH_2-CH_2-N(CH_3)_2$	Methiodide	EtOH	210–11°	55·5	6.09	55·1	5·80	>10,000 (2)	>5,000 (2)
10	$5-OC_7H_7$	$-CH_2-CH_2-N(CH_3)_2$	Ethiodide	EtOH	198–9°	55·7	5·81	56·0	6·06	>20,000 (2)	>10,000 (2)
11	$5-OC_7H_7$	$-CH_2-CH_2-N(C_2H_5)_2$	HCl	$EtOH/EMK/Et_2O$	171–2°	70·8	7·22	70·2	7·61	>10,000 (1)	470 ± 60 (4)
12	$5-OC_7H_7$	$-CH_2-CH_2-N(C_3H_7)_2$	HCl	EtOH	209–11°	71·7	7·65	71·4	8·10	>20,000 (1)	614 ± 80 (3)
13	$5-OC_7H_7$	$-CH_2-CH_2-N(isoC_3H_7)_2$	HCl	EtOH/EMK	215–18° (dec)	71·3	7·96	71·4	8·10	>10,000 (1)	867 ± 183 (2)
14	$5-OC_7H_7$	$-CH_2-CH_2-N(C_4H_9)_2$	HCl	EtOH/EMK	220–2° (dec)	72·1	7·81	72·4	8·52	>20,000 (1)	>20,000 (1)
15	$5-OC_7H_7$	$-CH_2CH_2N$ 	HCl	EtOH	227–9° (dec)	67·4	6·23	67·6	6·77	>20,000 (1)	>5,000 (2)

16	5-OH	$-CH_2-CH(CH_3)-NH_2$ (5-Hydroxy-α-methyltryptamine)	Creatinine sulphate HCl	(S) (C)							2 ± 0 (2)	1·7 ± 0·2 (5)
17	$5-OC_7H_7$	$-CH_2-CH(CH_3)-NH_2$	HCl	(S)							> 20,000 (1)	63 ± 11 (2)
18	$5-CH_3$	$-CH_2-CH_2-NH_2$ (5-Methyltryptamine)	HCl	(C)							173 ± 80 (3)	620 ± 180 (3)
19	$5-CH_3$	$-CH_2-CH(CH_3)-NH_2$ (5,α-Dimethyltryptamine)	HCl	(C)							1,300 ± 17 (3)	22·7 ± 1·5 (4)
20	$5-OCH_3$	$-CH_2-CH_2-NH_2$ (5-Methoxytryptamine)	HCl	(Cu)							8 ± 1·7 (3)	39 ± 9·7 (3)
21	$5-OCH_3$	$-CH_2-CH(CH_3)-NH_2$ (5-Methoxy-α-methyltryptamine)	HCl	(V)							4·3 ± 1·4 (3)	1·4 ± 0·25 (3)
22	$1-CH_3$	$-CH_2-CH(CH_3)-NH_2$	HCl	(S)							1,350 ± 450 (2)	69 ± 14 (3)
		$-CH_2-CH_2-NH_2$ (Tryptamine)	HCl	(I)							210 ± 30 (4)	933 ± 33 (3)
	5-OH	$-CH_2-CH_2-NH_2$ (5-Hydroxytryptamine)	Creatinine sulphate								1	1

bufotenine methiodide and ethiodide were obtained by alkylation of bufotenine base [3-(2-dimethylaminoethyl)-5-hydroxyindole] at room temperature.

The bufotenine synthesized, even after repeated crystallization, had a much lower melting point (125 to 126°) than that recorded by Wieland, Konz, and Mittasch (1934) and Speeter and Anthony (1954), namely 146 to 147°. Stoll *et al.* (1955), however, recorded m.p. 138 to 140°. As our material was distilled satisfactorily (b.p. 166 to 167°/10^{-2} mm. Hg pressure) and gave a reasonable analysis and fumarate, we suggest that it is a different crystalline modification. The bufotenine prepared by us was compared on the rat fundus strip preparation with a sample of bufotenine used by Gaddum *et al.* (1955). The two specimens were equiactive.

Preparations and Experimental Procedures.—These were identical with those previously described (Barlow and Khan, 1959) except that, in the initial qualitative tests on the rat uterus, no attempt was made to produce repeated contractions with all the compounds.

Results

Effects on the Isolated Rat Uterus Preparation

These were similar in character to those obtained with the tryptamines and can be divided into: (1) synergism with 5-hydroxytryptamine, tryptamine, and acetylcholine, (2) antagonism of 5-hydroxytryptamine and tryptamine, and (3) stimulant action. The effects of higher concentrations, which produced repeated contractions and profound depression, were not studied in any detail.

Synergism.—In our previous paper (Barlow and Khan, 1959), we interpreted this phenomenon as an addition, and showed that the presence of a small concentration of 5-hydroxytryptamine, which did not in itself produce any visible effect, caused increased responses to 5-hydroxytryptamine itself and to tryptamine and acetylcholine. We have found that a paeneliminal concentration (5×10^{-7}) of acetylcholine behaved similarly in that it enhanced the responses to 5-hydroxytryptamine, tryptamine, and acetylcholine.

Further evidence that the effect is little, if any, more than would be expected from simple addition was obtained by adding a dose of 5 ng. of 5-hydroxytryptamine at the same time as 100 ng. of acetylcholine. The contraction produced (Fig. 1) was only slightly larger than those produced either by 10 ng. of 5-hydroxytryptamine or by 200 ng. of acetylcholine acting alone. It was certainly less than the contractions produced by 20 ng. of 5-hydroxytryptamine or by 400 ng. of acetylcholine. This experiment was

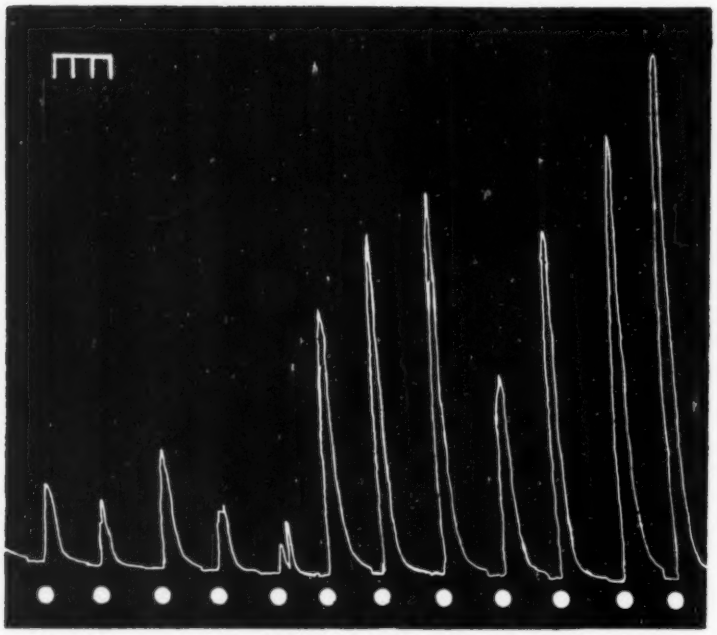


FIG. 1.—Isolated rat uterus preparation. Synergism between acetylcholine and 5-hydroxytryptamine. Contractions are shown in response to 10, 20, and 40 ng. of 5-hydroxytryptamine (**10**, **20**, and **40**) and to 200, 400, and 800 ng. of acetylcholine (**200**, **400**, and **800**). The response to a combined dose of 5 ng. of 5-hydroxytryptamine plus 100 ng. of acetylcholine (**5+100**) is not significantly greater than that to 10 ng. of 5-hydroxytryptamine or to 200 ng. of acetylcholine alone. Similarly the response to a combined dose of 10 ng. of 5-hydroxytryptamine plus 200 ng. of acetylcholine (**10+200**) is not significantly greater than that to 20 ng. of 5-hydroxytryptamine or to 400 ng. of acetylcholine alone. Bath vol., 2 ml. Time, min.

repeated, using twice the doses, with the same result, and also with tryptamine (1 μg. and 2 μg.) in place of 5-hydroxytryptamine. This type of addition was shown by all our compounds which possessed stimulant, but not antagonistic, activity.

Antagonism of 5-Hydroxytryptamine and Tryptamine.—As in previous work (Gaddum *et al.*, 1955; Barlow and Khan, 1959), it was necessary to allow 1 hr. for the full development of the antagonistic effect, and the reversal of this effect after washing took about the same time. The antagonistic activities of the compounds are shown in Table II. When some antagonists were present in high concentrations, the responses of 5-hydroxytryptamine did not increase as the concentration of 5-hydroxytryptamine was increased. In these circumstances the drug ratio cannot properly be determined; it appears to be very high and varies with the concentration of antagonist. This situation has been described by Gaddum *et al.* (1955), who call this type of block "unsurmountable." High concentrations of some of the compounds also depressed the responses to acetylcholine. There was no evidence, however, that they antagonized 5-hydroxytryptamine more than tryptamine (Table IIB).

5-Benzyloxy-3-(2-dialkylaminoethyl)-indoles all seemed to have the same order of activity although there was an increase up the series to the dipropyl compound (No. 12). The dibutyl compound (No. 14) was not very soluble and in the highest concentration tested (about 6×10^{-5} M) did not have any effect. The corresponding 5-hydroxy compound (No. 7) had quite marked effects and was the only 5-hydroxy compound which antagonized 5-hydroxytryptamine. The most active antagonist was 3-(2-aminopropyl)-5-benzyloxyindole (No. 17), but even this was less active than 5-benzyloxygramine.

Stimulant Action—The stimulant activity of the compounds is shown in Table I. The dose/response curves were all parallel to those of 5-hydroxytryptamine (and tryptamine) except those obtained with 3-(2-dibutylaminoethyl)-5-hydroxyindole (No. 7). This compound was the only one which definitely combined antagonistic and stimulant properties on this preparation (compare with 3-(2-dimethylaminoethyl)indole, Barlow and Khan, 1959) and in high concentration (10^{-5} M) usually produced repeated contractions and subsequent profound depression.

In one experiment 3-(2-aminopropyl)-1-methylindole (No. 22) antagonized 5-hydroxytryptamine, but in a second experiment (in the same concentration, 3×10^{-6} M, but on a different preparation) produced synergism with 5-hydroxytryptamine. In yet another experiment the same concentration produced a contraction by itself. Although this substance, therefore, combined antagonistic and stimulant properties, the stimulant effects predominated and masked the antagonistic ones (compare with 3-(2-diethylaminoethyl)indole, Barlow and Khan, 1959). Like 3-(2-dibutylaminoethyl)-5-hydroxyindole, this compound (No. 22) easily produced repeated contractions of the preparation but, after these, the tissue was more sensitive to 5-hydroxytryptamine for about 10 min. In this respect it resembled the analogues of tryptamine previously studied.

The most active compound was 3-(2-aminopropyl)-5-hydroxyindole (No. 16), and the next most active were 3-(2-aminopropyl)-5-methoxyindole (No. 21) and bufotenine (No. 1). Activity in the series of 3-(2-dialkylaminoethyl)-5-hydroxyindoles declined as the length of the alkyl group was increased, in contrast to the variation of activity with structure in the series of 3-(2-dialkylaminoethyl)indoles. The 5-methyl group and, to a much greater extent, the 5-benzyloxy group, reduced the stimulant activity. The quaternary salts tested were inactive as were also, although these are not included in Table I, the glyoxylamides, (5-benzyloxyindol-3-yl)glyoxyldimethylamide and (2-methylindol-3-yl)glyoxyldimethylamide, which are intermediates in the synthesis of the tryptamines.

Effects on the Isolated Rat Fundus Strip Preparation

Antagonism.—A few of the compounds, in low concentrations, antagonized the effects of tryptamine, and often, to a greater extent, of 5-hydroxytryptamine. This is shown in Table III, which includes results obtained with 5-benzyloxygramine and 5-benzyloxy-3-(2-dimethylaminoethyl)indole not reported in the previous paper. On this preparation, 5-benzyloxygramine was a less potent antagonist than 5-benzyloxy-3-(2-dimethylaminoethyl)indole or even than 3-(2-dimethylaminoethyl)indole itself (Table III). The action of 5-hydroxytryptamine on this preparation differed from that on the rat uterus in that it was much less sensitive to antagonism by 2-bromolysergic acid diethylamide and by 5-benzyloxygramine. For instance, on the rat uterus a concentration of 10^{-8} M-2-bromolysergic acid diethylamide produced an effect roughly comparable with that of a 10^{-7} M solution on the rat fundus strip. Similarly 5-benzyloxygramine, in a concentration of 9×10^{-7} M, gave effects on the rat uterus comparable with those of a concentration of 2×10^{-5} M on the rat fundus strip. On the other hand 3-(2-dimethylaminoethyl)indole in a concentration of 10^{-5} M produced a dose ratio for 5-hydroxytryptamine of 10 on the rat uterus, whereas it produced the same effect on the rat fundus strip in a concentration of 3×10^{-7} M. The antagonistic effects of 5-benzyloxy-3-(2-dimethylaminoethyl)indole also appeared to be greater on the rat fundus than on the rat uterus. On the rat fundus strip, 3-(2-dimethylaminoethyl)indole and 5-benzyloxy-3-(2-dimethylaminoethyl)indole were about equiactive whereas on the rat uterus the latter was more powerful.

TABLE II

ANTAGONISTIC ACTIVITY ON THE RAT UTERUS AT 30°

‡ Included from previous work for comparison (Barlow and Khan, 1959). † Indicates unsurmountable block (see text). * Indicates potentiation. In the drug ratio experiments the ratio of the concentration of the agonist to the concentration of the antagonist when the response was 50% of the initial maximum was used and the antagonist was allowed to act for 1 hr.: all concentrations are expressed in terms of molarity. In the dose ratio experiments the ratio is the number of times the concentration of the agonist must be increased in order to produce a 50% response after an antagonist has been allowed to act on the preparation for 1 hr. It should obviously increase with increasing the concentration of antagonist. The values for the dose ratio and drug ratio in the two parts of the Table must not be confused. It is only possible to proceed to determine the drug ratio if a value above 5 can be obtained for the dose ratio.

A. Drug Ratio Experiments using 5-Hydroxytryptamine

No.	Compound	Conc. (M)	Drug Ratio (Mean ± S.E.)	No. of Expts.
8	5-Benzyloxy-3-(2-dimethylaminoethyl)indole	90×10^{-7}	0·066 ± 0·008	4‡
11	5-Benzyloxy-3-(2-diethylaminoethyl)indole	7 21 55	0·074 0·093 0·47†, 9·25†	1 1 2
12	5-Benzyloxy-3-(2-dipropylaminoethyl)indole	5·2 13 26	0·095 0·107 ± 0·069 0·095	1 2 1
13	5-Benzyloxy-3-(2-di-isopropylaminoethyl)indole	6·5 13	0·076 ± 0 0·57†, 0·95†	2 2
15	5-Benzyloxy-3-(2-morpholinoethyl)indole	13 26	0·06 0·03	1 1
17	3-(2-Aminopropyl)-5-benzyloxyindole	7·8 32 78	0·16 0·27 0·12	1 1 1
7	3-(2-Dibutylaminoethyl)-5-hydroxyindole	30 75	0·10 ± 0·035 0·054	2 1

B. Dose Ratio Experiments

No.	Compound	Conc. (M)	Dose Ratio		
			5-Hydroxytryptamine	Tryptamine	Acetylcholine
12	5-Benzyloxy-3-(2-dipropylaminoethyl)-indole	$5\cdot2\times10^{-7}$	4	2	1
		13	20 4	27 8	1 1
		26	20	40	1
13	5-Benzyloxy-3-(2-di-isopropylamino-ethyl)indole	6·5	4 20	2 20	1 2
15	5-Benzyloxy-3-(2-morpholinoethyl)-indole	13 26	4 4	2 4	1 1·5
17	3-(2-Aminopropyl)-5-benzyloxyindole	7·8	5 4	8 3	1·5 1
		32	37	50	2
7	3-(2-Dibutylamino-ethyl)-5-hydroxy-indole	30	10 20	5 10	0·3* 0·6*
		75	20	25	0·5*

Although the dose ratios obtained in some instances were enough to justify attempts to measure the drug ratio, this was not done.

TABLE III

ANTAGONISTIC ACTIVITY ON THE RAT FUNDUS STRIP AT 37°

† Indicates potentiation. ‡ Indicates that these results confirm our previous estimates with this compound.

No.	Compound	Conc. (M)	Dose Ratios			
			5-Hydroxy-trypt-amine	Trypt-amine	5-Methyl-trypt-amine	Acetyl-choline
8	5-Benzyloxy-3-(2-dimethyl-aminoethyl)-indole	6×10^{-7}	10	2	—	< 1†
			10	2	—	< 1†
	5-Benzyloxy-gramine	18	2·5	1	—	1
		36	5	2	—	1
			5	2	—	1
		130	15	2	—	1
		180	27	8	—	2
		360	100	20	—	1
	3-(2-Dimethyl-aminoethyl)-indole‡	3·3	10	< 1†	—	1
			10	1·5	—	1
	2-Bromolysergic acid diethyl-amide	1·2	75	10	15	1
		2·5	60	8	8	1
		3·7	400	20	20	2

Estimation of the drug ratio requires the production of a maximal response, which on this tissue would render the preparation insensitive for a considerable time.

Stimulant Action.—All the compounds (except 5-benzyloxy-3-(2-morpholinoethyl)indole and the intermediate glyoxylamides mentioned above) stimulated the preparation. Those compounds listed above as antagonists showed this effect at concentrations greater than those which produced antagonism. In low concentrations the substances produced a gradual shifting of the base-line but, as was found with the tryptamines, there was no apparent synergism with 5-hydroxytryptamine, such as was seen on the rat uterus. There was no sign of any potentiation of tryptamine by these compounds (compare with 3-(2-dimethylaminoethyl)-2-methylindole, Barlow and Khan, 1959). The stimulant activity is summarized in Table I.

The dose/response curves of all the compounds, except those containing a 5-benzyloxy group, were parallel to those of 5-hydroxytryptamine (and tryptamine). The 5-benzyloxy compounds did not give a maximal response, although at lower concentrations the curves were parallel to those of 5-hydroxytryptamine, and it was at these concentrations that the equipotent molar ratios were determined.

5-Hydroxytryptamine, tryptamine, 3-(2-aminopropyl)-5-hydroxyindole (No. 16), 5-methoxytryptamine (No. 20), and 5-methyltryptamine (No. 18) caused contractions which reached a maximum within 90 to 120 sec. and which passed off after washing and stretching for about 1 min. The 5-benzyloxy compounds and 3-(2-aminopropyl)-1-methylindole (No. 22) required 2 or 3 min. for completion of the contraction, and 30 to 35 min. for stretching and recovery. The remaining compounds fell between these two extremes.

The most active compounds were 3-(2-aminopropyl)-5-hydroxyindole (No. 16) and 3-(2-aminopropyl)-5-methoxyindole (No. 21). The next most active were bufotenine and 3-(2-di-isopropylaminoethyl)-5-hydroxyindole. The variation of structure and activity in the series of 3-(2-dialkylaminoethyl)-5-hydroxyindoles was slightly different on the rat fundus from what it was on the rat uterus. Relative to the others, the dipropyl (No. 5) and di-isopropyl (No. 6) compounds were more active on the rat fundus than on the rat uterus.

In two instances (Nos. 19 and 21), substances containing a methyl group in the α position relative to the amino group were more active than the simple aminoethyl derivatives (Nos. 18 and 20), but this is not true for 5-hydroxytryptamine and 3-(2-aminopropyl)-5-hydroxyindole (No. 16). This result confirms the work of Vane (1959). The 5-methoxy compounds are again, as on the rat uterus, more active than their 5-methyl analogues.

Of the 5-benzyloxy compounds, the 3-(2-aminopropyl) member (No. 17) was the most active, but potency in the series, though declining in the higher members, did not follow the same pattern as in the corresponding 5-hydroxy compounds. As these compounds did not give a maximal response, we wished to see if they acted on the same receptors as 5-hydroxytryptamine. We therefore added simultaneously a 5-benzyloxy compound in a concentration which produced the biggest response obtainable (we used 6×10^{-5} M of the 3-(2-dimethylaminoethyl) compound (No. 8) or 5×10^{-5} M of the 3-(2-diethylaminoethyl) compound (No. 11)) and a dose of 5-hydroxytryptamine (100 ng. in a 5 ml. bath) which produced by itself a bigger effect than the 5-benzyloxy compound. In these circumstances (Fig. 2) the response was smaller than that to the dose of 5-hydroxytryptamine alone. When a dose of acetylcholine (200 μg.) was used in place of the 5-hydroxytryptamine, there was no depression of the response. This suggested that the 5-benzyloxy compounds occupied the same receptors as 5-hydroxytryptamine but not those acted on by acetylcholine.

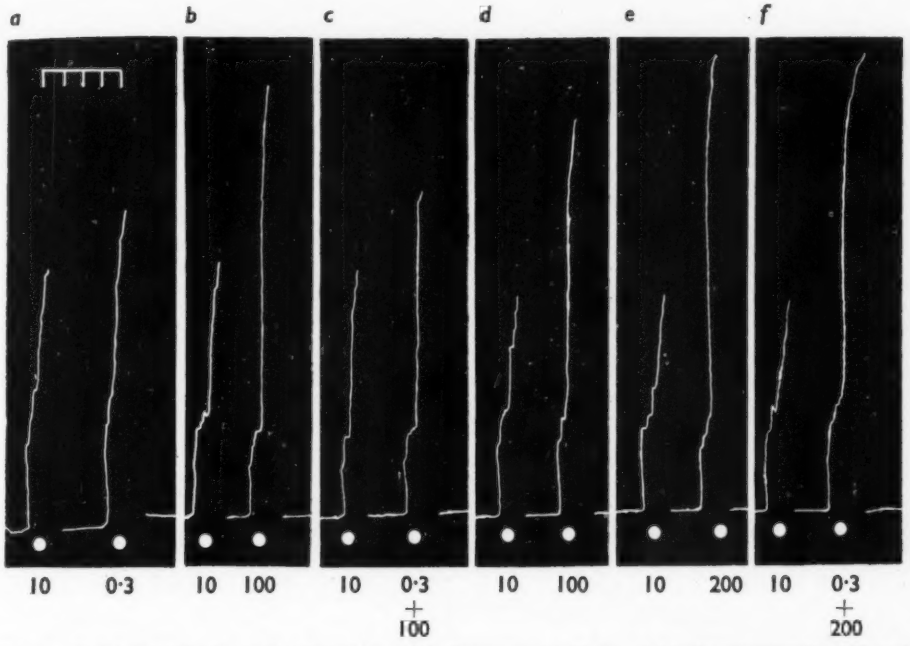


FIG. 2.—Isolated rat fundus strip preparation. (*a*), Responses to 10 ng. of 5-hydroxytryptamine (**10**) and 0.3 μMoles of 5-benzyloxy-3-(2-dimethylaminoethyl)indole (**0.3**) at time 12.28. (*b*), Responses to 10 ng. and 100 ng. of 5-hydroxytryptamine (**10** and **100**) at 14.05. (*c*), Responses to 10 ng. of 5-hydroxytryptamine (**10**) and a combined dose of 0.3 μMoles 5-benzyloxy-3-(2-dimethylaminoethyl)indole *plus* 100 ng. of 5-hydroxytryptamine (**0.3+100**) at 14.20. (*d*), Responses to 10 ng. and 100 ng. of 5-hydroxytryptamine at 15.45. (*e*), Responses to 10 ng. of 5-hydroxytryptamine (**10**) and to 200 μg. of acetylcholine (**200**) at 16.15. (*f*), Responses to 10 ng. of 5-hydroxytryptamine (**10**) and to a combined dose of 0·3 μMoles 5-benzyloxy-3-(2-dimethylaminoethyl)indole *plus* 200 μg. of acetylcholine (**0.3+200**). The results suggest that the 5-benzyloxy compound acts at the same receptors as 5-hydroxytryptamine but not at those acted upon by acetylcholine. Time, min.

Differentiation Between the Actions of Tryptamine and 5-Hydroxytryptamine

We again obtained evidence of differentiation between the antagonism of 5-hydroxytryptamine and of tryptamine on the rat fundus strip (Table III) but not on the rat uterus (Table IIB). 5-Benzyloxygramine and 5-benzyloxy-3-(2-dimethylaminoethyl)indole (No. 8) behaved like 2-bromolysergic acid diethylamide (and the compounds previously described, Barlow and Khan, 1959) in antagonizing 5-hydroxytryptamine more than tryptamine. Contractions produced by 5-methyltryptamine, however, unlike those produced by dimethyltryptamine and dipropyltryptamine, but like those produced by tryptamine, were much more resistant to the action of 2-bromolysergic acid diethylamide (Table III).

DISCUSSION

Antagonistic activity was confined to only a few compounds, those containing a 5-benzyloxy group and 3-(2-dibutylaminoethyl)-5-hydroxyindole (No. 7). Even the most active of these was not very powerful on the rat uterus, and on the rat fundus strip only 5-benzyloxy-3-(2-dimethylaminoethyl)-indole (No. 8) was an antagonist. On the rat fundus, however, the latter was more active than 5-benzyloxygramine and this draws attention to the different sensitivity of the two preparations. For activity as an antagonist of 5-hydroxytryptamine this difference is revealed by the fact that greater concentrations of 2-bromolysergic acid diethylamide and of 5-benzyloxygramine were needed on the rat fundus strip, whereas smaller concentrations of 5-benzyloxy-3-(2-dimethylaminoethyl)indole (No. 8) were needed on the rat fundus strip than on the rat uterus. Also, nearly all the antagonists (including 2-bromolysergic acid diethylamide and 5-benzyloxygramine) were more effective against 5-hydroxytryptamine than against tryptamine on the rat fundus strip whereas they do not differentiate between the two on the rat uterus.

Variations of activity with structure are also quite different with the two preparations. In the 5-hydroxy compounds, in particular, stimulant activity on the rat fundus strip after dropping from bufotenine (No. 1) to the diethyl compound (No. 4) increased again in the dipropyl and di-iso-

propyl compounds (Nos. 5 and 6); this is reminiscent of the maximum in activity on this preparation at the dipropyl compound in the simple tryptamines. With the rat uterus the relationships between structure and activity of the 5-hydroxy compounds were quite different from those of the analogues which lacked the 5-hydroxyl group. Again, apart from the 5-hydroxy compounds, most of the substances were, relative to 5-hydroxytryptamine, much more active stimulants on the rat fundus strip than on the rat uterus (this was particularly noticeable with dipropyltryptamine). The stimulant activity of the 5-hydroxy compounds relative to 5-hydroxytryptamine, however, was not greatly different on the two preparations.

Although, therefore, the general effects of the compounds on the rat uterus and rat fundus strip were not dissimilar, there were substantial differences which were brought to light especially by the 5-hydroxy compounds. Vane (1959) has suggested that the 5-hydroxy compounds are not able to penetrate cells but act at the surface, whereas non-phenolic compounds can penetrate the cell and so are subject to the action, inside the cell, of amine oxidase. Vane (1959) has shown that homogenates of rat fundus strip will oxidize 5-hydroxytryptamine and tryptamine to about the same extent and explains in this way why an α-methyl group enhances the activity of 5-methoxy- and 5-methyl-tryptamines and of tryptamine itself, but not that of 5-hydroxytryptamine. It would seem from our results with the rat uterus that this tissue should be deficient in amine oxidase and we have found that the presence of iproniazid (Marsilid), in concentrations of up to 3×10^{-5} M and allowed to act for over 30 min., had no effect on responses to 5-hydroxytryptamine and tryptamine. In such a concentration Vane (1959) found that iproniazid potentiated the action of tryptamine on the rat fundus strip by a factor of more than 10 but had no effect on the responses to 5-hydroxytryptamine.

The activity of the compounds may well depend upon their actions (either as substrates or inhibitors) on amine oxidase as well as their actions on the tryptamine and/or 5-hydroxytryptamine receptors. We have no systematic information about the action of the compounds on the enzyme, nor is it clear whether there are separate tryptamine and 5-hydroxytryptamine receptors. Furthermore, we might expect that substances which antagonize 5-hydroxytryptamine at one site would do so at others, also, perhaps, centrally. Similarly substances which stimulate, say, the rat uterus might be expected to be stimulants at other sites. In fact our results with the rat uterus and rat fundus strip do not justify extending the character of the action from one site to another. Until information has been obtained about all these points, we do not feel justified in speculating further about the actions, peripheral or central, of these compounds.

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UPTAKE OF INJECTED [^{14}C]ADRENALINE IN DENERVATED AND IN NORMALLY INNERVATED SUBMAXILLARY GLANDS OF THE CAT

BY

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Radioactive adrenaline was injected into cats after one submaxillary gland had been denervated, either by section of the chorda-lingual nerve or by excision of the superior cervical ganglion. No indication was found that the supersensitivity caused by denervation was due to a greater uptake of adrenaline in the denervated gland. Stimulation of the chorda tympani greatly increased the amounts of radioactive adrenaline taken up by normally innervated submaxillary glands.

Denervation of an excitable structure leads to an increased sensitivity to chemical agents. It has been suggested that this is caused by the development of a greater affinity towards drugs. Thus this increased response could be due to the chemical agent being taken up to a greater extent in the denervated structure. This hypothesis can be put to experimental test by using a substance which can be detected in small amounts. In the present series of experiments adrenaline labelled with ^{14}C was injected into cats, and the amounts present in normal and previously denervated glands at the height of secretion were estimated.

METHODS

Animal Experiments.—Cats weighing 2.5 to 3.5 kg. were used. About three weeks before the acute experiment the right chorda-lingual nerve was cut or the right superior cervical ganglion was excised. For the acute experiments, the animals were first anaesthetized with ether and then chloralose (80 mg./kg.). Glass cannulae were introduced into the submaxillary ducts. The glands were isolated, being connected to the animal only by the artery, veins, and duct. Drugs were injected either intravenously through a cannula in the femoral vein or intra-arterially through a cannula in the lingual artery. The intra-arterial injections (0.1 ml.) were made during 10 sec. In the case of intra-arterial injections, the venous outflow from the gland was drained off by a cannula introduced into the jugular vein. (±)-Adrenaline was injected and the drops of saliva falling from the cannulae in the salivary ducts were recorded on a smoked drum. A suitable dose giving a submaximal response was chosen. The time lag between the injection and the maximal rate of flow of saliva was noted. Later, injections of (±)-[^{14}C]adrenaline were made. After an intravenous injection both glands were removed simultaneously after an interval determined earlier in the same experiment. After an intra-arterial injection, the gland was similarly removed after a period determined earlier with non-radioactive adrenaline. In the case of intra-arterial administration, one gland was injected and removed first. Later the venous outflow from the remaining gland was sampled and finally this gland was injected and removed. The interval was the same for both sides.

Estimation of Radioactivity.—The activity of the whole gland was estimated, disregarding the fact that the gland loses in weight after denervation. The glands were minced in 5% trichloroacetic acid, homogenized and the volume brought up to 10 ml. with 5% trichloroacetic acid. The homogenate was centrifuged at low speed for 1 hr. The supernatant was decanted and the residue resuspended in trichloroacetic acid and centrifuged. This was repeated a third time. The three supernatants were mixed and excess of trichloroacetic acid was removed with ether. Finally, the supernatant was evaporated and the residue plated. The radioactivity was estimated in a flow counter with a background of 22 to 25 counts/min. Blood samples of 1 ml. were treated exactly as were the glands. (±)-[^{14}C]Adrenaline was obtained from Tracerlab Inc., Waltham, Mass., U.S.A.

RESULTS

Intravenous Injections

Non-stimulated Normal and Denervated Glands.—In a series of four cats with the right chorda-

lingual nerve cut 20 to 26 days earlier 20 μg./kg. of [^{14}C]adrenaline was injected intravenously. In Table I, which summarizes these experiments, it can be seen that the right (denervated) gland showed a higher radioactivity.

TABLE I

INTRAVENOUS INJECTIONS OF (±)-[^{14}C]-ADRENALINE IN CATS WITH THE RIGHT CHORDA-LINGUAL NERVE CUT

In this and subsequent Tables, the drops of saliva were counted after a previous injection of the same amount of unlabelled adrenaline.

No. of Days Denervated	Dose (μg./kg.)	Right Gland		Left Gland		Ratio of Counts/min. (Right/Left)
		Drops of Saliva	Counts/min.	Drops of Saliva	Counts/min.	
20	20	8·5	45	Trace	27	1·78
24	20	9	69	0·5	46	1·50
26	20	23	32	5	24	1·34
26	20	28	46	4	36	1·38
Mean		17	48	2·4	33	1·50

As the extraction procedure was not specific to adrenaline, radioactive metabolites of adrenaline might therefore contribute to the radioactivity estimated. Since the glands were removed shortly after the adrenaline had reached them, there is reason to believe that the radioactive metabolites of adrenaline present contributed only to a small extent to the estimated activity. The radioactivity detected was therefore considered to be a measure of the amount of adrenaline present at the moment of removal of the gland, and the greater activity exhibited by the denervated gland was interpreted as indicating that a greater amount of adrenaline was present. The results of the experiments in which adrenaline was administered intravenously did not necessarily suggest a greater uptake by the gland because the difference between the denervated and the normal gland could also be accounted for by a greater amount of blood containing radioactive adrenaline present in the denervated gland. Greater secretory activity in the denervated gland would have been accompanied by a greater vasodilatation.

Normal Stimulated Glands.—To test the effect of extreme vasodilatation on the radioactivity of normal glands in the cat, the chorda tympani on one side was electrically stimulated continuously during injection and removal of the glands. The outcome of three experiments of this kind is shown in Table II. Stimulation increased the amount of adrenaline present. To get information about the radioactivity of the blood, [^{14}C]adrenaline (in doses of 10, 20, and 20 μg./kg.) was injected into three cats (weighing 2.8, 2.7, and 3.2 kg. respectively). Blood was

TABLE II

INTRAVENOUS INJECTIONS OF (±)-[^{14}C]-ADRENALINE IN CATS WITH NORMAL SUBMAXILLARY GLANDS ON STIMULATION OF THE RIGHT CHORDA-LINGUAL NERVE

See Table I for explanation of drops of saliva.

Dose (μg./kg.)	Right Gland		Left Gland		Ratio of Counts/min. (Right/Left)
	Drops of Saliva	Counts/min.	Drops of Saliva	Counts/min.	
20	Trace	56	Trace	15	4·0
40	1	234	2	45	5·2
20	1·5	182	2	39	4·7
Mean					4·6

withdrawn from the left side of the heart at the same time as the removal of glands had been carried out in the earlier experiments. Activities of 30, 40, and 59 counts/min./ml. blood were found. Thus we can say that 20 μg./kg. of adrenaline gave about 60 counts/min./ml. and 40 μg./kg. about 120 counts/min./ml. The volumes of blood present in the stimulated glands calculated on the basis of these values would be 0.7, 1.6, and 2.4 ml. (Table II). The two last estimates of blood volume in the gland are obviously absurd, for the gland has only a weight of about 1 g. Some explanation for the increased radioactivity other than increased blood volume must therefore be found. Similarly an increase in the amount of tissue fluid in the gland could not account for the highly increased activity, even if adrenaline were carried only in the blood plasma and therefore values for tissue fluid should be only approximately one half of those calculated for blood. There must be a selective transport or uptake of adrenaline in some part of the stimulated and secreting gland probably involving the secreting cells.

Intra-arterial Injections

Section of the Chorda.—To minimize the error due to different amounts of radioactive blood present in the denervated and the contralateral gland, injections of adrenaline were made intra-arterially. In these experiments, the non-radioactive blood flowing during the time between the end of the injection and the removal of the gland could be supposed to wash out adrenaline not taken up by the tissues. To be able to compare the two sides, not simultaneously injected, the venous effluent from the gland first injected was drained off. The activity of blood emerging from the second gland was sampled before that gland was injected. When activity was found in this blood sample the experiment was discarded. The results obtained in cats with the right chorda-

TABLE III

INTRA-ARTERIAL INJECTIONS OF (±)-[^{14}C]-ADRENALINE IN CATS WITH THE RIGHT CHORDA-LINGUAL NERVE CUT

See Table I for explanation of drops of saliva.

No. of Days Denervated	Dose (μg.)	Right Gland		Left Gland		Ratio of Counts/min. (Right/Left)
		Drops of Saliva	Counts/min.	Drops of Saliva	Counts/min.	
24	0·5	8	48	1·5	46	1·04
24	0·5	5	11	Trace	20	0·55
30	0·5	9	27	1	27	1·00
21	0·5	19	57	1	68	0·84
18	0·5	5	51	3	104	0·49
17	0·5	12	136	1·5	73	1·86
15	0·5	7	83	1	73	1·14
19	0·5	16	82	2	107	0·77
Mean:		10	62	1·4	65	0·96

lingual nerve cut 15 to 30 days beforehand are shown in Table III, and it will be seen that the amounts of adrenaline taken up by the denervated and normally innervated gland were about the same. There was no indication that the denervated gland took up more active substance than the normally innervated one.

Excision of Superior Cervical Ganglion.—Not only section of the chorda but also excision of the superior cervical ganglion causes a supersensitivity in submaxillary glands. There is some indication that the mechanisms for the supersensitivity in glands denervated by these two procedures are different (Emmelin and Muren, 1951).

In four cats, the right superior cervical ganglion was removed; otherwise experimental conditions of the intra-arterial injection were the same as for the animals with the chorda cut. No indication of an increased uptake of radioactive adrenaline caused by the ganglionectomy was found (Table IV).

Dose/Activity Relation.—In work of this type it is of importance to use doses of adrenaline causing submaximal responses. For the animals with the right chorda cut, the dose given intra-arterially was 0.5 μg. of radioactive adrenaline, since higher doses caused almost maximal responses from the highly sensitized gland. After ganglionectomy, the dose chosen was in three out of four cats 1 μg. The mean of the uptake of the normal glands in the first series, with 0.5 μg. injected, was 65 counts/min., while the mean for the glands given 1 μg. was 73 counts/min. Into three normal glands 2 μg. was injected, giving activities of 107, 53, and 86 (mean 82) counts/min. In one cat, 10 μg. was injected, giving an activity of 126 counts/min. In Fig. 1, radioactivity and secretory responses have been plotted against the doses.

TABLE IV

INTRA-ARTERIAL INJECTIONS OF (±)-[^{14}]C-ADRENALINE IN CATS WITH THE RIGHT SUPERIOR CERVICAL GANGLION EXCISED

See Table I for explanation of drops of saliva.

No. of Days Denervated	Dose (μg.)	Right Gland		Left Gland		Ratio of Counts/min. (Right/Left)
		Drops of Saliva	Counts/min.	Drops of Saliva	Counts/min.	
24	1	3·5	46	2·5	107	0·43
21	1	1·5	67	Trace	58	1·15
23	0·5	5	23	1	36	0·64
20	1	5·5	94	3·5	54	1·75
Mean:						0·99

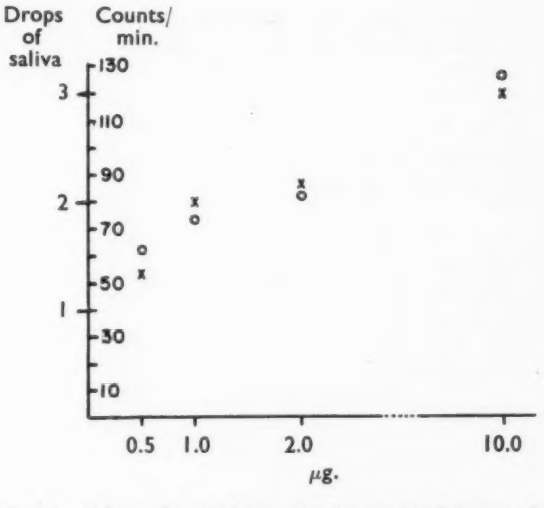

FIG. 1.—The number of drops of saliva produced from and the radioactivity in normally innervated submaxillary glands have been plotted against the dose of (±)-[^{14}C]adrenaline given intra-arterially. The values are means. **X**, Secretion; **O**, radioactivity.

DISCUSSION

When radioactive adrenaline was injected intravenously, it was found that the amount present in the glands, made supersensitive by previous section of the chorda, was greater than in the normally innervated glands. No such difference could be found when the injections were made intra-arterially. The difference after intravenous injections could be due to the greater amount of blood present in the denervated gland, this gland giving a greater secretory response. The difference in radioactivity shown in Table I could well be accounted for in this way. This conclusion was supported by the finding that, after intra-arterial injections, there was no increased radioactivity in the denervated gland

However, when an attempt was made to test this hypothesis further by comparing the activity of a resting and an electrically stimulated gland, considerably more radioactivity was found in the stimulated gland; this difference was larger than that to be expected from the presence of an increased amount of blood or extracellular tissue fluid. It was concluded that adrenaline moves into the gland cells when they are made to secrete by stimulation of the chorda tympani.

The radioactivity after intravenous injections was less than after intra-arterial injections even though the secretory response to adrenaline given was greater after intravenous administration (Tables I and II). This was probably due to the fact that the duration of action of the drug was longer after intravenous injection, the drug being continuously supplied to the gland.

No indication was found that sympathetically or parasympathetically denervated glands took up more adrenaline than normally innervated glands when the drug was given intra-arterially. It is possible, but unlikely, that there is much unspecific absorption and that a specific difference is obscured. It is of interest that in normally innervated glands when different doses were given intra-arterially and the response and uptake were plotted against the dose there seemed to be a parallelism between the secretory effect and the amount of adrenaline taken up (Fig. 1).

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PARALDEHYDE AND METHYLPENTYNOL AND GANGLIONIC TRANSMISSION

BY

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Paraldehyde and methylpentynol blocked transmission of nerve impulses through the superior cervical ganglion of the cat when the drugs were administered intra-arterially to the ganglion or intravenously using the nictitating membrane as an indicator. Electrical studies showed that concentrations of methylpentynol and paraldehyde which blocked transmission in the isolated rat superior cervical ganglion were without action on the preganglionic nerve fibre. In amounts which blocked transmission in the isolated rat ganglion, paraldehyde had no depolarizing activity directly on the ganglion cells and did not interfere with the depolarizing activity of added acetylcholine. The results suggest that the block in transmission of the impulse could be accounted for by a decrease in the release of acetylcholine from the preganglionic nerve terminals. In both species the block was reversible.

Paraldehyde and methylpentynol block neuromuscular transmission (Quilliam, 1955). Electrical studies showed that the blocking activity of both drugs could be accounted for if their action decreased acetylcholine release (Nicholls and Quilliam, 1956). In the present work, the effect of paraldehyde and methylpentynol on ganglionic transmission has been studied using, first, the nictitating membrane preparation in the cat and, second, the isolated superior cervical ganglion of the rat with electrical recording of the compound nerve action potentials in the pre- and post-ganglionic nerves. The experimental evidence showed that paraldehyde and methylpentynol could block ganglionic transmission reversibly in the cat and in the rat. The action of methylpentynol at the ganglion has been briefly reported (Quilliam, 1957).

METHODS

Nictitating Membrane Preparation in the Cat

Cats were anaesthetized with pentobarbitone sodium (Nembutal 30 mg./kg., intraperitoneally). The pre- and post-ganglionic nerves to the superior cervical ganglion were cleaned and prepared for stimulation in the usual manner. Rectangular pulses of 0.5 msec. duration at 10/sec. from an electronic stimulator were applied through platinum electrodes to stimulate the nerves. The preganglionic nerve was stimulated continuously and the response of the nictitating membrane on that side was recorded on a smoked paper. When necessary, the response of the preparation to postganglionic stimulation could be tested by electrodes applied to the postganglionic nerve. The voltages of the pulses used in each instance were slightly greater than those producing maximal retraction of the membrane. The lingual artery was prepared for intra-arterial injections of drugs into the ganglion during which the external carotid artery was clamped (Morrison and Paton, 1953).

The Isolated Superior Cervical Ganglion of the Rat

Rats were anaesthetized with urethane (1.2 g./kg. intraperitoneally). The superior cervical ganglion was exposed. The ganglion with its pre- and post-ganglionic nerves was removed from the rat and transferred to Krebs solution at room temperature (20°) and bubbled with 95% O_2 and 5% CO_2. The connective tissue sheaths investing the preparation were then removed.

For the electrical recordings, the ganglion was suspended horizontally between two insulated forceps in a bath filled with liquid paraffin B.P. previously bubbled with 95% O_2 and 5% CO_2. Platinum electrodes were used both for stimulating and recording and were applied to the surface of the preparation in fixed positions at the beginning of each experiment. The two stimulating electrodes (2 mm. apart) were placed as far proximally as was possible on the preganglionic trunk. Single electrical stimuli of sufficient voltage at 0.5 msec. duration were used to produce a maximal action potential in the preganglionic nerve. One pair of recording

electrodes (2 mm. apart) was placed on the preganglionic trunk to record the preganglionic nerve action potential. Between this pair of electrodes and the stimulating electrodes, the preparation was earthed. Another pair of recording electrodes (2 mm. apart) were placed upon the ganglion for recording ganglionic potentials and a further pair of electrodes (2 mm. apart) were applied to the postganglionic trunk as far distally as possible for recording the postganglionic nerve action potential. When recordings were being made, the appropriate pair of electrodes were selected by a switch assembly and the pulses picked up by them were led through a cathode follower, passed to a direct coupled amplifier (Copeland, 1952) and displayed on an oscilloscope equipped with a camera.

Whether bathed in Krebs solution or in liquid paraffin at room temperature, transmission was maintained satisfactorily for 8 to 10 hr.

For recording the depolarizing action of acetylcholine upon the ganglion, the preparation was set up vertically in a 60 ml. bath of Krebs solution at room temperature previously bubbled with 95% O_2 and 5% CO_2 with the postganglionic nerve lowermost and a moving fluid electrode technique was employed similar to that described by Fatt (1950) for the toe muscle of the frog. The present technique differed from that of Pascoe (1956) mainly in that the postganglionic trunk was lowermost. Neostigmine methylsulphate (Roche) 2.5×10^{-6} was added to the Krebs solution bathing the ganglion. The ganglion was exposed to acetylcholine for 2 min. and then washed thoroughly for 45 min.

Drugs

Paraldehyde B.P. and pure methylpentynol (British Schering) were used. They were dissolved in 0.9% sodium chloride solution for intra-arterial injection in the nictitating membrane preparation. As variation in the injection volume led to inconsistent results, the intra-arterial injections were always made in a total volume of 0.25 ml. containing the required quantity of drug.

In the studies of transmission through the isolated rat superior cervical ganglion, hexamethonium bromide (May and Baker) was dissolved in Krebs solution. Paraldehyde and methylpentynol were mixed by vigorous shaking in a stoppered vessel with medicinal liquid paraffin B.P. at room temperature before they were added to the bath containing the preparation. The liquid paraffin was bubbled with 95% O_2 and 5% CO_2 before mixing with the drug. For recording purposes it was an advantage to be able to dispense with aqueous solutions of drugs. Preparations in liquid paraffin produced satisfactory responses for at least as long as those bathed in Krebs solution.

In depolarization experiments, all drugs were dissolved in Krebs solution.

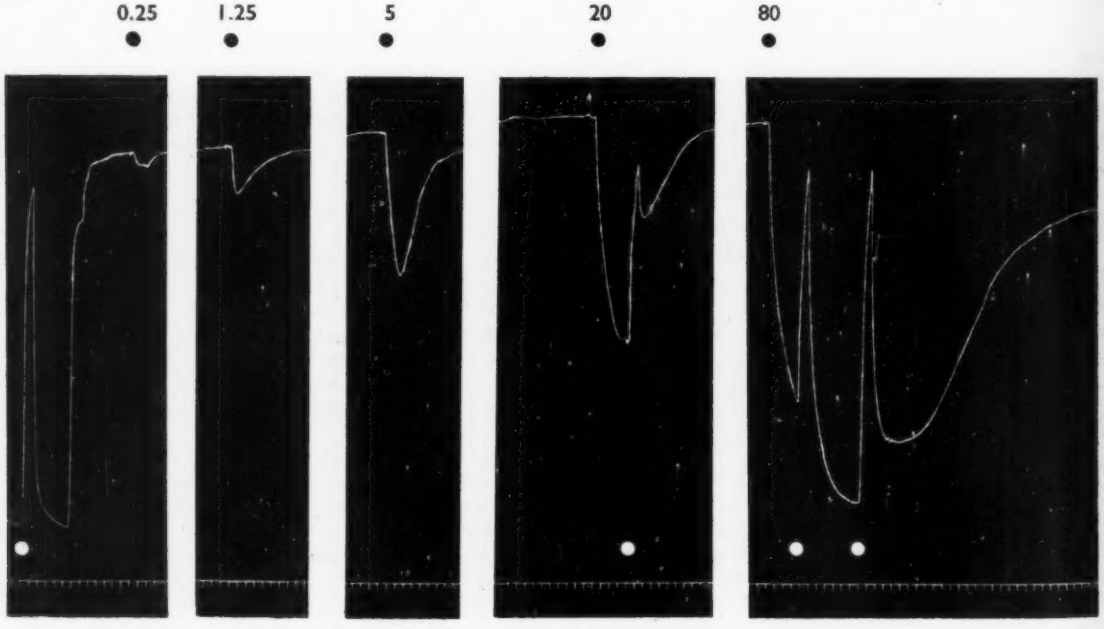

FIG. 1.—Record of response of the nictitating membrane in a cat (4.4 kg., pentobarbitone anaesthesia) to continuous stimulation of the preganglionic nerve to the superior cervical ganglion of that side for the period indicated by the black line below. At the white dots, the postganglionic nerve was stimulated for 30 sec. The numerals above the black dots indicate the quantities of paraldehyde in mg. contained in 0.25 ml. injections intra-arterially to the ganglion. At the time of maximal reduction of the retraction of the nictitating membrane following the last two paraldehyde injections postganglionic stimulation was fully effective. Time, 30 sec.

RESULTS

Nictitating Membrane Preparation in the Cat

Paraldehyde. — If paraldehyde was injected intra-arterially during continuous preganglionic stimulation, the response of the nictitating membrane was reduced and the reduction in the retraction increased with increasing dosage. With total injection volumes of 0.25 ml., 0.25 mg. of paraldehyde usually produced a just perceptible effect. A 50% reduction was caused by about 15 mg. and complete relaxation of the membrane occurred with about 80 mg. of paraldehyde. The block seemed to be ganglionic because post-ganglionic stimulation remained fully effective. Recovery from the blocking action of small and moderate quantities of paraldehyde was full and not unduly delayed. Large doses gave rise to delayed recovery of transmission which was not complete even after 1 hr. These effects are illustrated in Fig. 1.

The time course of the block with paraldehyde was not dissimilar from that seen with a concentration of hexamethonium giving a comparable degree of block.

Much larger doses of paraldehyde had to be given intravenously to produce a block in ganglionic transmission. In one experiment even after 1 ml. of paraldehyde B.P. administered over a period of 6 min. as an intravenous infusion, block was not quite complete. Postganglionic stimulation gave a maximal retraction of the membrane, but the response to preganglionic stimulation showed no sign of recovery 1 hr. later.

Methylpentynol.—With intra-arterial injections in a total volume of 0.25 ml., a reduction in the retraction of the nictitating membrane in response to continuous stimulation usually appeared with 0.25 mg. of methylpentynol. With increasing amounts of methylpentynol, there was an increasing reduction of the retraction. About 3 mg. caused a 50% reduction and 20 mg. usually produced complete or almost complete abolition of the retraction. With all but the largest doses of methylpentynol in this range, the block was much more rapid in onset and the recovery much quicker than with doses of paraldehyde producing comparable reductions in the retraction of the

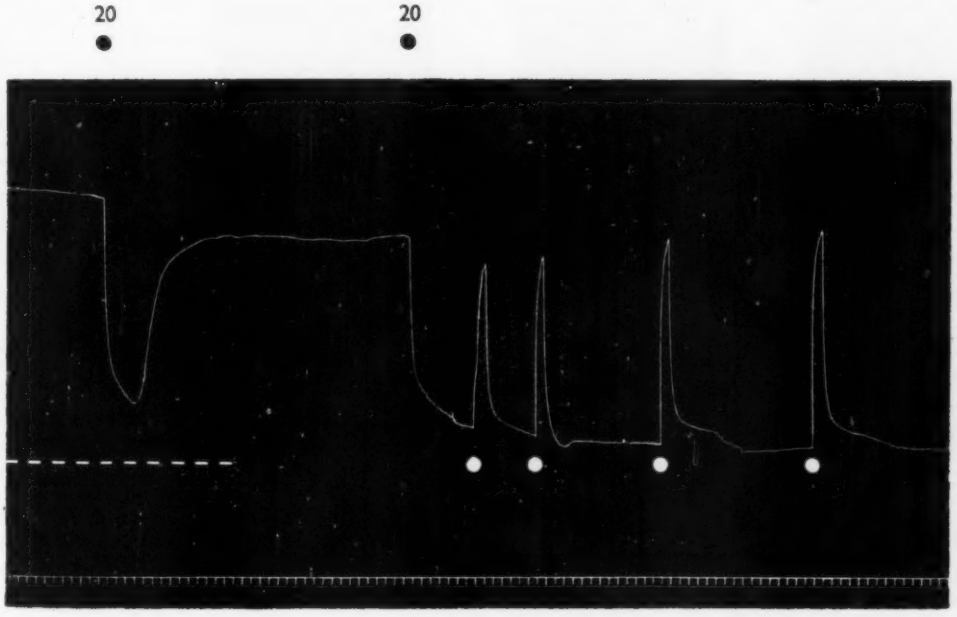

FIG. 2.—Record of response of the nictitating membrane in a cat (3.27 kg., pentobarbitone anaesthesia) to continuous stimulation of the preganglionic nerve to the superior cervical ganglion of that side for the period indicated by the black line below. At the white dots, the postganglionic nerve was stimulated for 30 sec. The numerals above the black dots indicate the quantities of methylpentynol in mg. contained in 0.25 ml. injections intra-arterially to the ganglion. The second injection of 20 mg. of methylpentynol produced almost complete relaxation of the membrane from which no recovery was observed 1 hr. later. The level of complete relaxation of the nictitating membrane is indicated by the horizontal broken white line. Time, 30 sec.

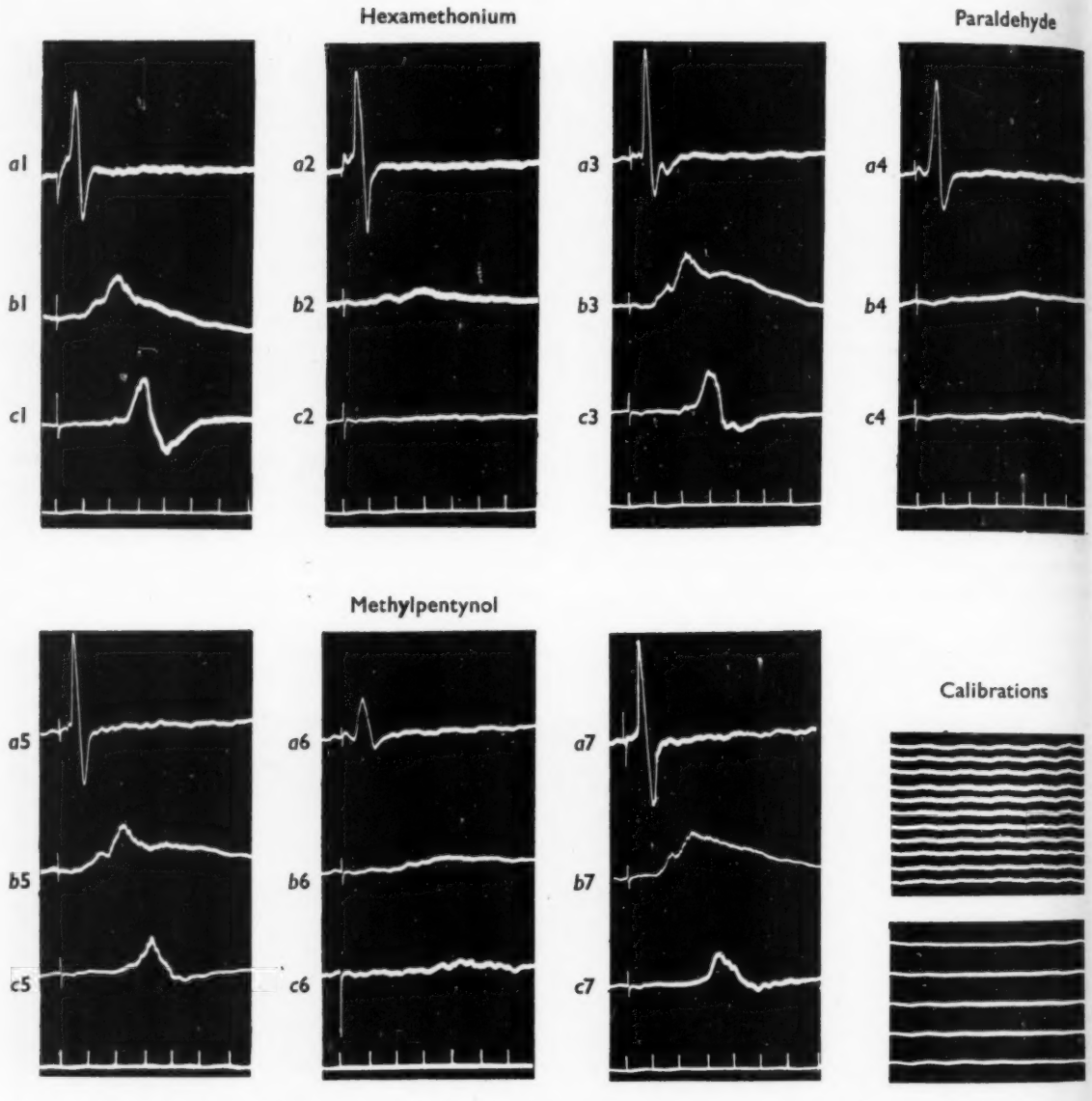

FIG. 3.—The isolated superior cervical ganglion of the rat bathed in liquid paraffin. Recordings of the electrical changes in the preganglionic nerve (series ***a1***, ***a2***, ***a3***, etc.), in the ganglion (series ***b1***, ***b2***, ***b3***, etc.) and in the postganglionic nerve (series ***c1***, ***c2***, ***c3***, etc.) after a single stimulus applied to the preganglionic nerve. See text for fuller explanation. When the ganglion was treated with 500 μg./ml. of hexamethonium in Krebs solution, the ganglionic potential was reduced (***b2***) and the postganglionic nerve action potential was abolished (***c2***) indicating a complete block of ganglionic transmission. After washing in Krebs solution, transmission through the ganglion was restored as is shown by the return of the ganglionic potential (***b3***) and the postganglionic nerve action potential (***c3***). Similar reversible blocks of ganglionic transmission are shown with paraldehyde (12 mg./ml.) and methylpentynol (4 mg./ml.) each of which was applied to the preparation mixed with liquid paraffin. With methylpentynol, the preganglionic action potential was reduced. Only a small increase in the amount of methylpentynol above that producing ganglionic block caused an effect on the preganglionic nerve. The blocking actions of both paraldehyde and methylpentynol were reversed by washing in liquid paraffin. Time, 20 msec. The upper calibration is a series of 100 μV. steps and applies to all records in the series indicated by the letter ***a*** and ***b*** and also to ***c6***. The lower calibration is in 1 mV. steps and refers to all records in the series labelled ***c*** except ***c6***.

membrane. With 20 mg. of methylpentynol, the recovery from the block was slower than with smaller quantities. After repeated intra-arterial injections of 20 mg. of methylpentynol, the block was sufficiently prolonged to test the response to postganglionic stimulation which was full or only slightly and temporarily impaired. There was little if any recovery of ganglionic transmission after methylpentynol block when two or more intra-arterial injections of 20 mg. had been given (Fig. 2).

When 0.5 to 1 ml. of pure methylpentynol was slowly administered intravenously, a prolonged block of ganglionic transmission occurred.

Electrical Responses Recorded from the Isolated Superior Cervical Ganglion of the Rat

Effects on Transmission Through the Ganglion.—Fig. 3 illustrates the electrical responses recorded from an isolated ganglion preparation bathed in liquid paraffin at room temperature when the preganglionic nerve was stimulated electrically. The nerve action potential in the preganglionic nerve is shown in Fig. 3*a1*. Potentials recorded from the preganglionic nerve are all denoted as Fig. 3*a1*, *a2*, *a3*, etc. The potential from the ganglion is depicted in Fig. 3*b1* ; all ganglion potentials are labelled Fig. 3*b1*, *b2*, *b3*, etc. The action potential recorded from the postganglionic nerve is shown in Fig. 3*c1*, all postganglionic potentials being designated Fig. 3*c1*, *c2*, *c3*, etc.

In the series Fig. 3*a1*, *b1*, and *c1*, the preganglionic nerve action potential arose after a brief latent period (*a1*). The ganglion potential (*b1*) developed after a longer latent period as might be expected from the time required for the impulse to be conducted down the preganglionic fibre and to be transmitted across the ganglionic synapse. The postganglionic potential (*c1*) arose later still as still more time had to elapse between the moment of stimulation (stimulus artifact) and the time at which the postganglionic impulse passed the recording electrodes on the postganglionic trunk.

If transmission of the nerve impulses across the ganglionic synapse was blocked then a reduction of the ganglionic potential (which contains components of electrical changes in the ganglion cells and in the postganglionic fibres arising therefrom) and a reduction or abolition of the postganglionic nerve action potential with partial or complete ganglionic block respectively would be observed. There should be no change in the nerve action potential recorded from the preganglionic nerve if block is confined to the ganglion.

Hexamethonium.—When the ganglion was soaked in Krebs solution containing 500 μg./ml. of hexamethonium bromide, a complete block of ganglionic transmission occurred, the postganglionic potential being abolished (Fig. 3*c2*) and the ganglion potential reduced (*b2*). The preganglionic action potential (*a2*) was slightly increased, possibly for the reasons given below. After washing the preparation thoroughly with Krebs solution, ganglionic transmission was completely restored (Fig. 3*b3* and *c3*).

For recording, Krebs solution was replaced with liquid paraffin. Changes in the film of Krebs fluid investing the nerve when the preparation was immersed in oil may account for the slight differences in recordings between washes with Krebs solution in spite of attempts to remove all the Krebs solution completely from the preparation with slips of blotting paper. Such differences are those seen when the records in Fig. 3*a1*, *a2*, *a3*, *a4*, *a5*, and *a7* are compared.

Paraldehyde.—The miscibility of paraldehyde with liquid paraffin enabled the ganglion to be exposed to various concentrations of this drug without resorting to aqueous solutions. In the experiment illustrated in Fig. 3, 12 mg./ml. of paraldehyde in liquid paraffin was required to produce a complete block of transmission through the isolated ganglion, the ganglionic potential being reduced (*b4*) and the postganglionic nerve action potential abolished (*c4*). Repeated washing in liquid paraffin restored ganglionic transmission (Fig. 3*b5* and *c5*).

Methylpentynol. — When the ganglion was bathed in liquid paraffin containing 4 mg./ml. of methylpentynol, the ganglion potential (*b6*) was reduced and postganglionic nerve action potential abolished (*c6*). There was some reduction in the preganglionic nerve action potential (*a6*). That this block of ganglionic transmission was reversible can be seen from Fig. 3*b7* and *c7* in which, after repeated washing in liquid paraffin, the ganglion and postganglionic nerve action potentials were restored to near their values at the beginning of the experiment.

It was usually possible to find a concentration of methylpentynol which blocked ganglionic transmission without diminishing the preganglionic nerve action potential. Only a slight increase in this concentration produced impairment of conduction in the preganglionic nerve, and the diminished height of the preganglionic nerve action potential illustrated in Fig. 3*a6* may be accounted for in this way. This effect was reversed by washing (Fig. 3*a7*).

Depolarization Experiments.—To find whether paraldehyde in concentrations producing complete ganglionic block depolarized the ganglion cells directly or interfered with the depolarizing action of acetylcholine on the ganglion cells, experiments with the moving fluid electrode technique were carried out. First, a concentration of acetylcholine required to produce about 50% of the maximal depolarization of the ganglion was determined. In one experiment, 25 μg./ml. of acetylcholine in the bath fluid produced 2 mV. depolarization in 2 min. Next, 4 mg./ml. of paraldehyde in Krebs solution was found to abolish the post-ganglionic response to preganglionic stimulation. Under these conditions, no depolarization of the ganglion was recorded and the depolarizing activity of 25 μg./ml. of acetylcholine added to the fluid bathing the ganglion was unimpaired, there being 2 mV. of depolarization after 2 min. Pascoe (1956) reported that the hyperpolarization of the isolated rat superior cervical ganglion after washing off acetylcholine reached a peak in 2 min. This finding was confirmed in the present work and it was observed that paraldehyde in a blocking concentration did not modify the hyperpolarization seen after removal of acetylcholine from the bath fluid.

Discussion

The results show that paraldehyde and methylpentynol can block ganglionic transmission in the cat and in the rat. Previous work has shown that these two drugs can block neuromuscular transmission in the frog and in the rat (Quilliam, 1955). The results of the electrical studies of neuromuscular transmission in the frog by Nicholls and Quilliam (1956) could be accounted for if paraldehyde and methylpentynol reduce the release of acetylcholine. Direct estimates of acetylcholine release from the hind-quarters of the frog when perfused with Ringer solution containing paraldehyde in concentrations which abolished neuromuscular transmission in this preparation appeared to confirm this suggestion (Nicholls and Quilliam, unpublished observations).

The experiments with the nictitating membrane preparation in the cat localized the action of paraldehyde and methylpentynol to the ganglion because postganglionic stimulation was fully effective when the drugs had blocked the response to preganglionic stimulation.

The results from the electrical studies on the isolated rat superior cervical ganglion showed that the two drugs in ganglion blocking concentrations had no action on the preganglionic nerve fibre.

Depolarization studies excluded actions of paraldehyde producing a direct depolarization of the ganglion cell and a competitive block because the depolarizing activity of added acetylcholine was unchanged. In these experiments the amount of paraldehyde used was sufficient to block ganglionic transmission.

Taken together these results suggest that paraldehyde and methylpentynol produce ganglionic block by reducing the release of acetylcholine from the preganglionic nerve terminals. In this connexion it is interesting to note that Marley and Paton (1959) have found that methylpentynol can reduce the output of acetylcholine from the perfused cat superior cervical ganglion and that a reduction of 50% or more of the acetylcholine output was associated with failure of transmission.

Recently much attention has been directed to methylpentynol. Marley and Vane (1958) developed methods by which they were able to study the distribution of the drug and its carbamate in body fluids and in tissues, and suggested that the slow metabolism of both compounds might lead to cumulative effects. Marley (1959) made a wide study of the pharmacology of methylpentynol and its carbamate. This author confirmed the ganglion blocking action of methylpentynol reported by Quilliam (1957) and showed that the drug could also depress mono- and poly-synaptic reflexes.

The experimental evidence already referred to that methylpentynol can block synaptic transmission and the clinical impressions that the drug may allay apprehension (Trotter, 1953; Bourne, 1954) have led to attempts to evaluate the effect of the drug on the central nervous system. Dicker, Steinberg, and Watson (1957) have obtained evidence that methylpentynol appeared to reduce fear in rats. Dicker and Steinberg (1957) attempted to assess the effect of methylpentynol in man. They showed that, although the compound depressed the autonomic reactions of the subject in response to a difficult motor task, it impaired performance and they considered that its mode of action differed from that of hexobarbitone. The ganglion-blocking activity of methylpentynol reported here could account for the depression of autonomic responses found by Dicker and Steinberg (1957).

It is of practical interest that drugs may be applied to the isolated rat superior cervical ganglion mixed with liquid paraffin which is a particularly suitable medium in which to record nerve and ganglion action potentials. However,

with the ganglion immersed in liquid paraffin, about three times the concentration of paraldehyde required in Krebs solution was needed to produce complete block. This difference might be accounted for by the oil/water partition coefficient of the drug.

It is with much pleasure that the author thanks Mr. D. F. J. Mason and Mr. D. A. Brown for help with the nictitating membrane experiments, and Mr. K. A. H. Didcock for technical assistance. He wishes to acknowledge the gifts of hexamethonium from Messrs. May & Baker, and of methylpentynol from Messrs. British Schering.

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PHARMACOLOGY OF METHYLPENTYNOL AND METHYLPENTYNOL CARBAMATE

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The pharmacology of methylpentynol and methylpentynol carbamate has been studied. The drugs depressed monosynaptic and polysynaptic reflexes, and exerted weak ganglion and neuromuscular blocking actions. They also produced transitory hypotension, and increase of aortic blood flow. Perfusion of the coronary arteries with these drugs led to slowing of the heart, diminished systolic amplitude, dysrhythmias, and increased ccronary flow. Respiration was stimulated with small and depressed with larger doses of both drugs. The two compounds diminished the response of the isolated guinea-pig ileum to drugs acting directly on muscle or through ganglia. The effect of the two substances was likened to that of other central nervous system depressants, particularly to that of ethanol.

The toxicology, hypnotic and anticonvulsive effects of methylpentynol (3-methyl-1-pentyn-3-ol, Oblivon) were described by P'An, Gardocki, Harfenist, and Bavley (1953), Marshall and Vallance (1955) and Gross, Tripod and Meier (1955). Similar studies of these properties of methylpentynol carbamate were made by Halpern (1956), by Halpern and Lehmann (1956) and by Barnes, McCrea, Marshall, Sheahan, and Walsh (1958). Although other pharmacological actions of methylpentynol have received less attention, some have been described by Margolin, Perlman, Villani, and McGavack (1951) and by Gialdroni and Grassi (1952). Quilliam (1955) studied the effect of methylpentynol and other hypnotic drugs on frog skeletal muscle. The present work is concerned with properties of methylpentynol and its carbamate other than anticonvulsant effects.

METHODS

Isolated Tissues.—In all experiments with isolated tissues, the animal was killed by a blow on the head and exsanguination. The tissue was rapidly removed and the dissection completed in cold 0.9% sodium chloride solution.

Kitten Hearts.—The coronary vessels were perfused with oxygenated Locke solution (NaCl, 9 g.; KCl 0.42 g.; $CaCl_2.6H_2O$, 0.472 g.; glucose 2.0 g.; $NaHCO_3$ 0.5 g.; water to 1,000 ml.) at 37° and at a constant pressure head. The Martin-Langendorff technique was used with the apparatus described by Baker (1951). Methylpentynol or methylpentynol carbamate, dissolved in the Locke solution, was injected into the aortic cannula from a Palmer slow injector. Coronary outflow was collected in a funnel draining into a graduated cylinder.

Frog Rectus.—The isolated rectus abdominis muscle of the frog was suspended in a modified Ringer solution (NaCl 6 g.; KCl 0.15 g.; $CaCl_2.6H_2O$ 0.3 g.; $NaHCO_3$ 0.5 g.; water 100 ml.) and bubbled with 5% CO_2 and 95% O_2. Contractures were recorded using a gimbal lever of magnification $\times 9$ giving an effective muscle load of 0.8 g.

Electrically Stimulated Guinea-pig Ileum.—The method of Paton (1955) using coaxial electrical stimulation was employed. The tissue was suspended in a 10 ml. bath of Krebs solution bubbled with 95% O_2 and 5% CO_2 at 35 to 37°. Rectangular pulses of 1.0 msec. duration and of sufficient strength to produce a maximal response to a single shock were applied to the electrodes, the intraluminal electrode being the anode. Contractions were recorded with a pendular auxotonic lever (Paton, 1957a) with a magnification of 8.

Isolated Rat Phrenic Nerve Diaphragm Preparation.—The preparation was set up in the manner described by Bülbring (1946) but with minor modifications. The tissue was suspended in 50 ml. of Tyrode solution bubbled with O_2 at 36 to 37°. Rectangular supramaximal pulses of 0.5 msec. duration were applied to the nerve at a frequency of 6/min. The stimulating electrode consisted of 2 rings of platinum wire 2 mm apart embedded in one end of a polythene tube (5 mm. i.d.). The upper end of the tube was above the surface of the fluid in the bath and the nerve was drawn into the tube (filled with Tyrode solution) by a thread. Muscle contractions were recorded with a semi-isometric lever.

Other Methods.—The cats were anaesthetized with chloralose (80 mg./kg.) after induction by ethyl chloride and ether. In some experiments, spinal cats were prepared by the method of Kosterlitz, Krayer, and Matallana (1955) or by pithing. Rabbits were anaesthetized with urethane (1 g./kg., 25% solution). Rectal temperatures were maintained at 38°. When methylpentynol or its carbamate was injected intraperitoneally into guinea-pigs, the carotid artery was usually cannulated during the anaesthesia produced by the drug, 5 ml. blood was withdrawn and laked with distilled water, and the brain was then removed. If, however, the anaesthetic effect of the drug was insufficient, the animal was given ethyl chloride and ether before carotid cannulation. Because of the volatility of methylpentynol, blood and tissue samples were stored in sealed tubes until required for estimation.

Blood Pressure.—Recordings were made from the left carotid artery on a kymograph using a mercury manometer connected to a siliconed glass cannula filled with heparin-saline.

Blood Flow.—The density flowmeter described by Dawes, Mott, and Vane (1953) was connected in series with the lower abdominal aorta below the renal vessels.

Respiration.—The apparatus described by Paton (1949) was used for cats and rabbits. In man, the volume of respiratory expired air was measured by an integrating motor pneumotachograph (Wolff, 1958), % O_2 and CO_2 composition being determined by the method of Scholander (1947).

Neuromuscular Transmission.—The tension of the twitch of the tibialis anterior muscle of the cat (and in one experiment of both tibialis and soleus muscles) was recorded with an isometric steel myograph, the preparation being mounted on the Brown-Schuster myograph stand. The sciatic nerve was tied centrally, above the point of stimulation and above the point of entry of its blood supply. The muscle was excited by slightly supramaximal shocks at a frequency of about 6/min. applied to the sciatic nerve through shielded platinum electrodes. In some experiments, the muscle was tetanized with shocks at a rate of 50/sec. for a duration of 1 sec. in every 12 sec. Intravenous injections were made either into a femoral or jugular cannula. Retrograde arterial injections were given into the contralateral common iliac artery which was ligatured just below the cannula. Infusions were given by a Palmer slow injector.

Nictitating Membrane.—Records were made of the contracture of the nictitating membrane (after enucleation of the eyeball) in response to stimulation of the preganglionic or postganglionic portions of the cervical sympathetic trunk which had been separated from the vagus and cut centrally. Platinum wire electrodes were applied to the nerve to be stimulated by supramaximal rectangular pulses of 0.5 msec. duration at a frequency of 10/sec., either continuously or for 30 sec. periods at regular intervals. Infusions were made into the right femoral vein and retrograde arterial injections into the stump of the ipsilateral lingual artery. In another experiment, the responses of both nictitating membranes were recorded. The isotonic levers were adjusted so that equal shortening gave equal movements of the writing point. The loading weight was 4.0 g. and the magnification seven-fold. The same parameters of stimulation were used although the stimulating circuits on the two sides were independent. The animal was artificially ventilated and the chest opened by splitting the sternum. The drugs were injected into the right internal mammary artery with the distal aorta occluded during continuous stimulation of the right preganglionic and the left postganglionic cervical sympathetic trunks. In this way the effects of arterial injection to both nictitating membrane preparations could be studied simultaneously.

Suprarenal Gland. — The blood pressure of eviscerated pithed cats was recorded. The peripheral end of the sectioned nerve to the left suprarenal gland was stimulated with rectangular pulses of 0.5 msec. duration and of 20 volts at a frequency of 10/sec. for 30 sec. which resulted in maximum output of sympathetic amines (Marley and Paton 1958, unpublished observations). Injections of methylpentynol or its carbamate into the right femoral vein were made 45 sec. prior to the period of stimulation.

Knee Jerk.—The jerk was elicited by a solenoid tapping the quadriceps tendon of a cat every 5 sec. and recorded on a kymograph by a lever attached to the ankle by a thread.

Stomach Contraction Following Vagal Stimulation. —In cats, the oesophageal and pyloro-duodenal junctions were tied. The stomach was cannulated on the greater curvature, washed out and filled with 60 ml. of saline and movements were recorded on a smoked paper. Both vagi were stimulated in the neck by supramaximal rectangular pulses of 0.5 msec. duration at a frequency of 5/sec. for 60 sec. which gave maximal contraction of the stomach (Burge and Vane, 1958).

Gastric Secretion Induced by Histamine.—Cats were deprived of food, but not water, from the previous evening as Black, Fisher, and Smith (1958) have shown that histamine-induced secretion may decline and even cease in animals fed shortly before the experiment. Under chloralose anaesthesia, the abdomen was opened in the midline, the pyloro-duodenal junction occluded (leaving the pancreatico-duodenal arteries intact) and a glass cannula tied into the body of the stomach near the greater curvature. The abdominal wound was closed around the cannula and the animal turned on its side. The oesophagus was also tied off to prevent drainage of saliva and mucus into the stomach, the vagi being left intact. The stomach was then washed out with warm saline and the juice collected in graduated centrifuge tubes. Histamine acid phosphate (15 μg. base/min.) was

infused from a Palmer slow injector connected to the right femoral vein. The animals were given artificial respiration throughout the experiment to minimize respiratory variation.

Estimate of Acute Toxicity.—Male and female albino mice of Schofield strain were employed weighing about 15 g. Food and water were not withheld prior to the experiment and the drugs were injected intraperitoneally. It was not possible to give the doses in equal concentrations because of the different solubility of the two drugs. The methylpentynol was injected in concentrations of 40 mg./ml. in 0.9% saline, the carbamate in a concentration of 20 mg./ml. in slightly warm saline.

Measurement of Dissociation Constants.—Solutions were made up using distilled water which had been boiled and allowed to cool in an atmosphere of nitrogen. 5.0 ml. of a 1.0 mg./ml. solution of either methylpentynol or the carbamate was pipetted into a tube and 1.0 ml. of 0.1 N-HCl added to bring the *p*H below 2.0. The electrodes of a Pye *p*H meter, connected to an ink recorder, were lowered into the solution, together with two lengths of syringe needle tubing. Nitrogen was bubbled through one of these at a rate to give adequate stirring, the other being connected to a micrometer syringe containing 5 N-NaOH (carbonate free). The syringe piston was advanced at a constant rate by a velodyne motor, the speed adjusted to deliver 0.02 ml. of alkali/min. As the *p*H of the solution was continuously recorded by the pen writer on moving paper, the titration curve was drawn directly. Neither methylpentynol nor methylpentynol carbamate was found to dissociate.

Oil/Water Partition Coefficient.—Olive oil B.P. was shaken with a buffer solution of *p*H 7.0 to remove any acidity. The emulsion was separated by centrifugation and the oil then washed in a similar manner with distilled water. Methylpentynol (5.0 mg.) or methylpentynol carbamate (5.0 mg.), in the form of a 1.0 mg./ml. solution in distilled water, was added to 5.0 ml. of olive oil, treated as above, or to 5.0 ml. of oleyl alcohol, and shaken mechanically for 60 and 120 and 240 min. at room temperature. The emulsion was then separated by centrifugation in capped tubes and an aliquot from both the water and the oil phases distilled as described below for estimation of the drugs, the carbamate being hydrolysed with 5.0 ml. 3N-NaOH which gave a mean recovery of 80% added material. Methylpentynol carbamate may be hydrolysed using alkali when dissolved in distilled water, olive oil or oleyl alcohol, but acid hydrolysis must be used if the drug has to be distilled from body fluids or tissues. The $C_{olive\ oil}/C_{water}$ was in all cases 1/1. The $C_{oleyl\ alcohol}/C_{water}$ was 2.2/1. for methylpentynol after 1 hr., rising to 2.6/1. after 4 hr. shaking; that for the carbamate 2.8/1. at 1 hr., and 3.8/1. after 4 hr.

Estimation of Methylpentynol or Methylpentynol Carbamate.—Methylpentynol or its carbamate was estimated by the method of Perlman, Sutter and Johnson (1953) as modified by Marley and Vane (1958). Methylpentynol, or methylpentynol carbamate after previous treatment with 2 to 3 ml. of 3N sulphuric acid, was distilled from the specimen into N/40 ammoniacal silver nitrate in which methylpentynol combined quantitatively with silver to form a precipitate. After centrifugation, the supernatant fluid was decanted and the silver remaining in solution was titrated with N/40 potassium thiocyanate. Standard curves were obtained for both drugs by adding them directly to N/40 ammoniacal silver nitrate, centrifuging and titrating the silver remaining in solution with N/40 potassium thiocyanate. The mean recovery of methylpentynol from cat blood was 95% and of methylpentynol carbamate 65%. The accuracy of the method was within 0.01 mg. for methylpentynol, but for the carbamate concentrations in blood or tissue recovery experimental results have been corrected for a 65% recovery, then given to the nearest 0.05 mg./g. or ml.

For injection and infusion, pure methylpentynol was dissolved in 0.9% saline. The methylpentynol carbamate was also dissolved in slightly warmed saline for infusion experiments (up to 20 mg./ml.) but, for some single injections, had to be dissolved in 65% aqueous propylene glycol in order to minimize the volume. When comparing the effects of the two drugs, they were injected in the same dilution, since it is known (Mellanby, 1919) that dilute solutions of alcohol have less physiological effect than strong solutions containing the same amount of alcohol. For the infusion experiments in man, the methylpentynol was dissolved in dextrose solution (5.0 g. methylpentynol and 5.0 g. dextrose in 100 ml. distilled water). The two drugs conformed to the following analytical standards. *Methylpentynol:* the refractive index was between 1.4305 to 1.4315 at 21°, and not less than 90% of the substance distilled between 118° and 123°. It contained no more than 10 p.p.m. of lead, 1 p.p.m. of arsenic, and there was no more than 0.05% of residue. *Methylpentynol carbamate:* this contained not less than 96.0% and not more than the equivalent of 101.1% of $C_7H_{11}O_2N$. The freezing point was not less than 51.5° and the melting point about 52°. It contained no more than 5 p.p.m. of arsenic, 20 p.p.m. of lead, and no more than 0.1% sulphated ash.

Drugs Used.—Adrenaline bitartrate, noradrenaline bitartrate, histamine acid phosphate, acetylcholine chloride, atropine sulphate, physostigmine sulphate, nicotine hydrogen tartrate, decamethonium iodide, barium chloride and gallamine triethiodide. Weights refer to the base in the instances of adrenaline, noradrenaline and histamine and to the salts of all the other compounds.

Results

Acute Toxicity.—The number of deaths and the number of mice which remained asleep 24 hr. after intraperitoneal injection of either methylpentynol or methylpentynol carbamate are

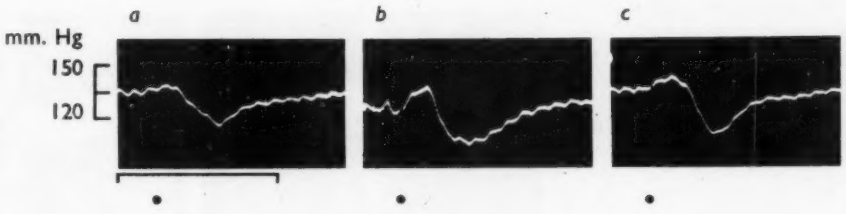

FIG. 1.—Blood pressure of a cat (2.5 kg., chloralose anaesthesia). Intravenous injections in (*a*) of 50 mg. of methylpentynol, in (*b*) of 1 μg. of histamine, and in (*c*) of 50 mg. of methylpentynol carbamate. Time, 30 sec.

shown in Table I. The approximate LD50 from the combined results for both sexes was 320 mg./kg. for the carbamate and 550 mg./kg. for methylpentynol yielding an equipotent molar activity of 2.5 for methylpentynol carbamate compared to 1 for methylpentynol. Allowing for the small numbers of animals employed, there was no significant difference between the sexes in their susceptibility to the drugs. Attention is drawn to the number of animals still asleep 24 hr. after drug administration.

TABLE I

TOXICITY OF METHYLPENTYNOL AND METHYLPENTYNOL CARBAMATE

Number of male or female mice dead (D) or asleep (S) 24 hr. after the intraperitoneal injection of methylpentynol or methylpentynol carbamate in the doses stipulated. 10 mice used with each dose.

Drug	Dose (mg./kg.)	Male		Female	
		D	S	D	S
Methylpentynol	400	0	0	1	2
	467	3	5	0	0
	533	4	2	5	3
	600	7	2	6	1
	800	10	—	10	—
Methylpentynol carbamate	267	1	2	3	2
	300	4	3	4	5
	333	5	3	5	1
	367	6	4	5	5
	400	7	1	9	1

Blood Pressure.—Four experiments were done. Single intravenous doses of 25 mg. or greater of either drug were followed by an immediate and transitory fall of blood pressure without bradycardia, an effect which was seen in anaesthetized, spinal, or pithed animals. The response to a single intravenous injection of 50 mg. of methylpentynol matched and followed the same time course as that to 1 μg. of histamine, the hypotension being slightly less than that due to the injection of 50 mg. of methylpentynol carbamate. Recovery occurred in approximately 30 sec. (Fig. 1). Intravenous infusion of either drug caused a prolonged hypotension (Figs. 4 and 5).

Heart.—In two experiments, the perfusion of the coronary system of the isolated kitten heart (Fig. 2) with methylpentynol or its carbamate led initially to a slowing of the heart, a decrease in amplitude of ventricular systole, the appearance of occasional extrasystoles, followed by their

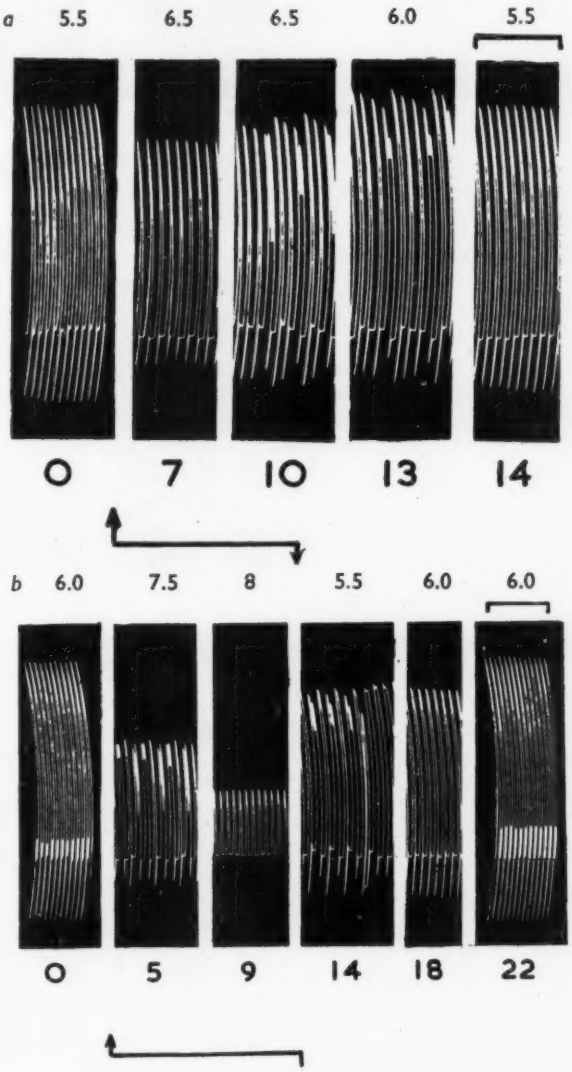

FIG. 2.—(*a*) Isolated kitten heart preparation. The numerals below indicate the time in min. at which records were taken after adding methylpentynol (0.5 mg./min.) to the perfusion fluid for 10 min. The numerals above indicate coronary flow in ml./min. Systole, upwards. Time, 5 sec. (*b*) Isolated kitten heart preparation. Recordings as in (*a*) but perfusion with 1 mg./min. of methylpentynol carbamate for 10 min.

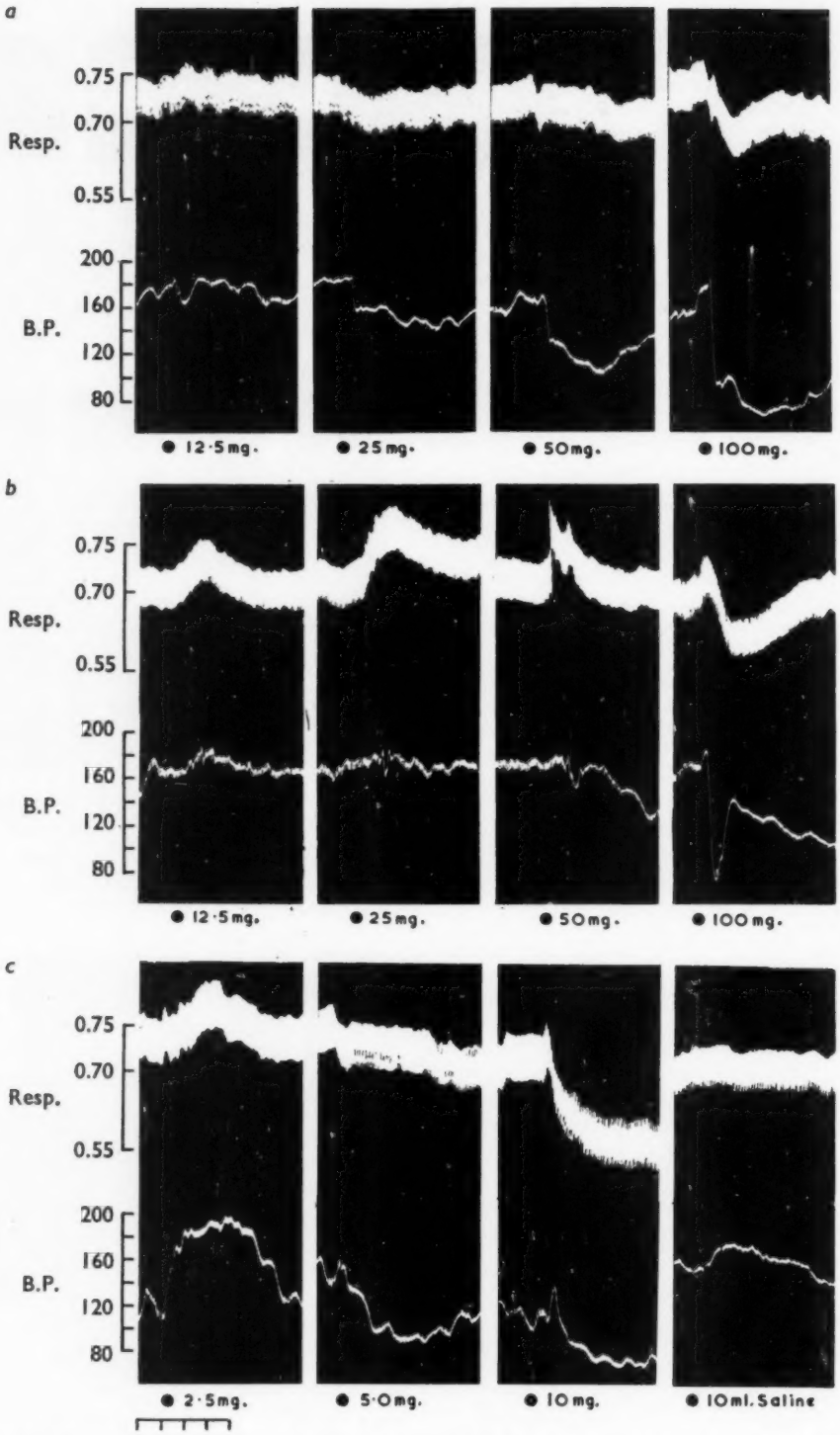

FIG. 3.—Records of respiration (**Resp.**) and blood pressure (**B.P.**) in a cat (2.5 kg., chloralose anaesthesia). Respiration scale, min. vol. in l., blood pressure scale mm. Hg. Responses after single intravenous injections of (*a*) methylpentynol, (*b*) methylpentynol carbamate, and (*c*) sodium pentobarbitone (dose in mg.) and, in lowest right-hand record, 10 ml. saline. Time, min.

regular occurrence after every fourth, third, or second beat. At this stage, the ventricular response might return to a normal regular rate but with a marked reduction of systolic amplitude (Fig. 2*b*). During recovery, these phenomena recurred but in reverse order. Coronary flow was increased by between 15 and 30% during perfusion of the drugs.

These responses arose with a perfusion concentration for methylpentynol of 5 mg. in 58 ml. (1/1,160 solution) and for methylpentynol carbamate of 10 mg. in 75 ml. (1/750 solution).

Blood Flow in the Hind Limbs of the Cat.—In one experiment, the injection of either methylpentynol or its carbamate into the arterial system produced vasodilatation, similar increases of blood flow occurring with 6 mg. of pentobarbitone sodium, 10 mg. of methylpentynol carbamate, and 12 mg. of methylpentynol. Vasoconstriction was never seen.

Respiration

Single Injections.—In four experiments, all but the smallest doses of both drugs were respiratory depressants. Thus the intravenous injection of 5 mg./kg. (total 12.5 mg.) or 10 mg./kg. (total 25 mg.) of either drug had no effect on respiration or produced slight increase of the respiratory rate and minute volume (Fig. 3). Doses in excess of 20 mg./kg. led to a reduction of minute volume, falling from about 725 ml. with 40 mg./kg. (total 100 mg.) of methylpentynol carbamate to about 600 ml./min., and with the same dose of methylpentynol from 750 ml. to just less than 700 ml./min., full recovery taking some 15 min. The diminution of minute volume was associated with a fall of blood pressure. A small increase of respiratory minute volume was seen with the intravenous injection of 1 mg./kg. of sodium pentobarbitone, but a marked reduction of minute volume followed the administration of 4 mg./kg. of sodium pentobarbitone, the fall being from 700 to 550 ml. air/min.

Infusions.—Considerably greater depression of respiration occurred in two cats during infusions of either drug (Figs. 4 and 5). Thus after the infusion of 1,940 mg. methylpentynol in 76 min. (blood concentration, 1.25 mg./ml. of methylpentynol) the respiratory minute volume had fallen from about 0.5 to about 0.1 l. The infusion of 1,800 mg. of methylpentynol carbamate in 105 min. led to complete arrest of respiration (corrected blood concentration of the carbamate, 0.5 mg./ml.). There was a reduced response to preganglionic stimulation of the nictitating membrane. There was also a 60% reduction of the knee jerk with both drugs. In two experiments on man, intravenous infusion over 15 min. of 1.0 g. methylpentynol was associated with a brief increase of respiratory rate and volume (Table II).

TABLE II

THE EFFECT ON RESPIRATION IN MAN OF INTRAVENOUS INFUSIONS OF SALINE OR METHYLPENTYNOL

		Before Infusion	During Infusion	10 min. after End of Infusion
Infusion of 20 ml. of saline during 15 min.	Respiratory rate/min.	16	12	16
	Vol. of expired air l./hr.	344·0	290·0	307·0
	O_2% in expired air	16·4	17·2	16·2
	CO_2 in expired air	3·9	3·4	3·0
Infusion of 1·0 g. of methylpentynol in 20 ml. during 15 min.	Respiratory rate/min.	16	24	17
	Vol. of expired air l./hr.	397·5	457·5	401·3
	O_2% in expired air	16·8	17·3	16·7
	CO_2% in expired air	4·4	3·9	4·4

Action on Smooth Muscle

Isolated Guinea-pig Ileum.—The changes in responses to acetylcholine and to coaxial electrical stimulation of the guinea-pig ileum with methylpentynol (M.W.98), methylpentynol carbamate (M.W.141), sodium pentobarbitone (M.W.248), and ethanol (M.W.46) was studied next. Fig. 6 shows the response of the electrically stimulated ileum to methylpentynol and its carbamate. Dose/response curves are given in Figs. 7 and 8. As the slopes differed to such an extent, no accurate relative potencies could be calculated. However, approximate relative potencies were determined by comparing doses required to reduce the maximal response to coaxial stimulation, or the response to acetylcholine by 50%, the dose of acetylcholine being adjusted to give a half of the maximal effect. In two experiments on the electrically stimulated guinea-pig ileum, the equipotent molar dose of sodium pentobarbitone was 1, and those for methylpentynol carbamate, 4.8; methylpentynol, 13.8; and ethanol, 1,071.5. In one experiment on the ileum stimulated by acetylcholine, the approximate equipotent molar ratios where sodium pentobarbitone was 1 were methylpentynol carbamate, 9.1; methylpentynol, 18.8; and ethanol, 1,084.6.

The effects of methylpentynol and its carbamate were also studied in 3 experiments on the guinea-pig ileum stimulated by $BaCl_2$ or nicotine, half maximal response to acetylcholine being matched by contractions of similar height produced by

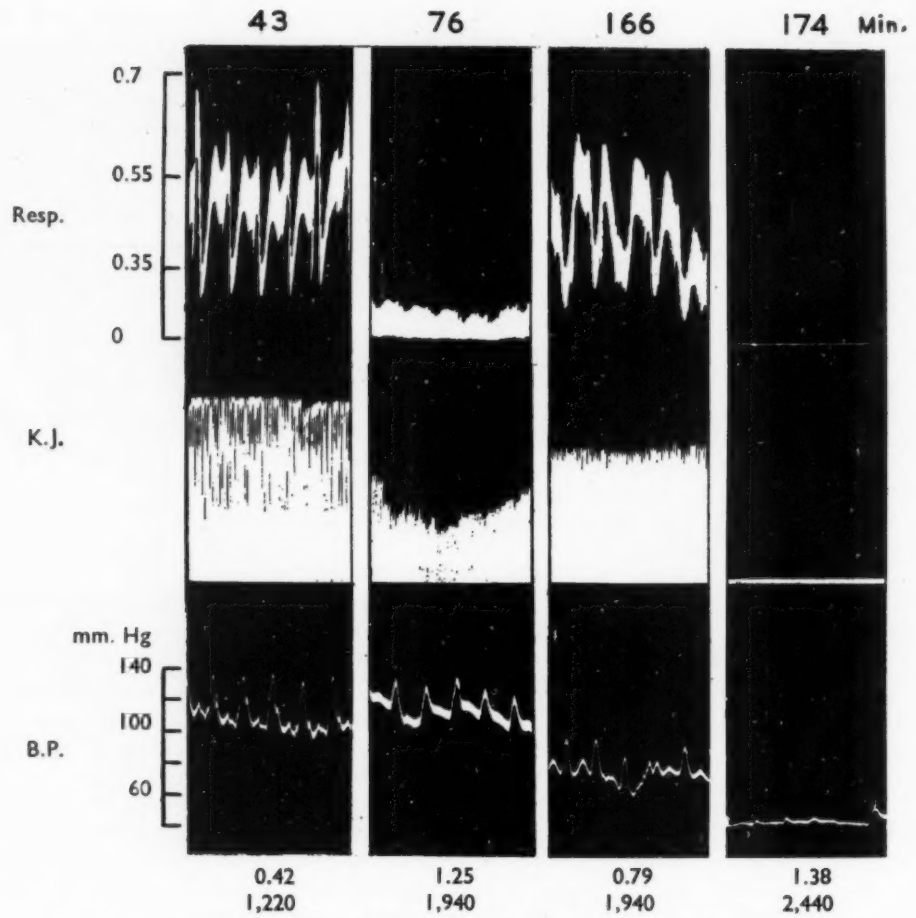

FIG. 4.—Records of respiration (**Resp.**), knee jerk (**K.J.**), and blood pressure (**B.P.**) in a cat (4.1 kg., chloralose anaesthesia). Records of **Resp.** and **B.P.** as in Fig. 3. The numerals in the upper row below the records indicate the whole blood concentration (in mg./ml.) and those in the lower row the total quantity of infused methylpentynol (in mg.).

the other two substances. The effect of methylpentynol or carbamate was tested only after the enhanced contraction to acetylcholine immediately succeeding the addition of $BaCl_2$ or nicotine had returned to normal. Methylpentynol carbamate was more potent on an equimolecular basis than methylpentynol (1 : 2.5) in reducing the acetylcholine response, which took longer to regain its full contraction height than after methylpentynol. In this dose ratio, the drugs produced approximately identical reductions (50 to 60%) of the acetylcholine and $BaCl_2$ contractions (Fig. 9). The nicotine response was reduced by 75 to 80%. In the presence of hyoscine (10^{-7}), the same doses of methylpentynol and the carbamate produced an 80 to 90% diminution of the acetylcholine and $BaCl_2$ contractions.

Contraction of the Stimulated Stomach in situ.—In 2 experiments, single intravenous doses of either drug up to 200 mg. (60 mg./kg.) or 800 mg. of ethanol (240 mg./kg.) had no effect on intestinal tone nor on the response of the saline-filled stomach to vagal stimulation.

Action on Striated Muscle

Isolated Frog Rectus Abdominis Muscle.—In 5 experiments, methylpentynol and its carbamate in high concentrations (4×10^{-3}) elicited contracture of the frog rectus equivalent in height to that produced by 10^{-7} of acetylcholine (Fig. 10*a*). As neither drug dissociates, this was not a *p*H effect, nor was it an osmotic effect, as the addition to the bath of a solution of sucrose (42×10^{-3}) approximately three times the molar strength of a solution of methylpentynol (4×10^{-3})

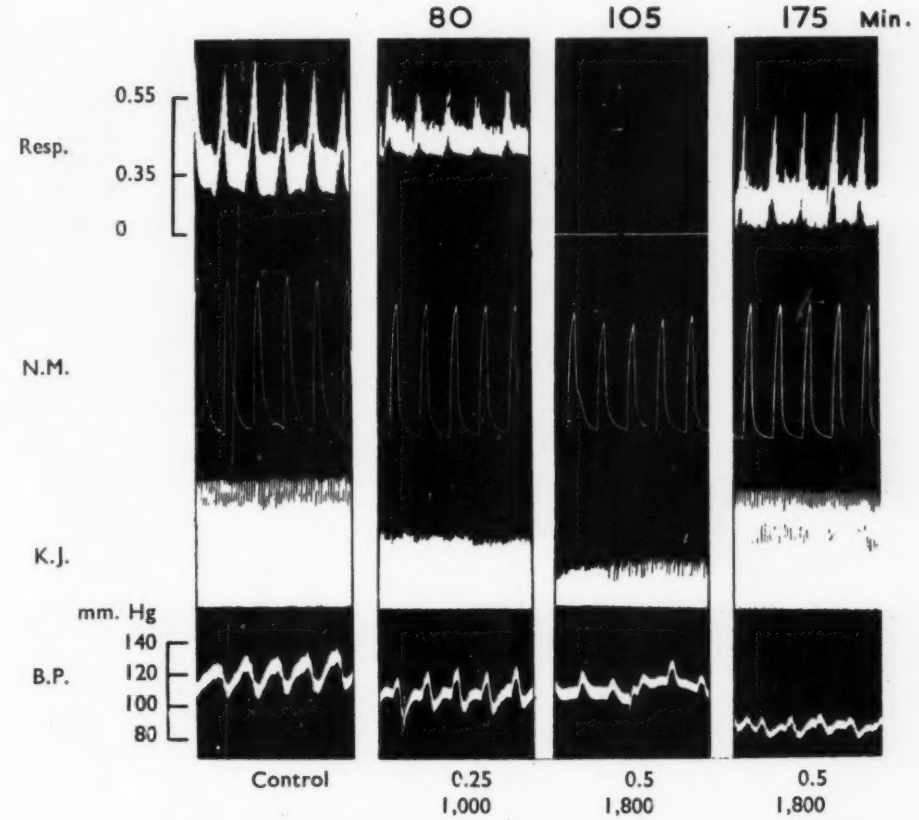

FIG. 5.—Records of respiration (Resp.), nictitating membrane stimulated preganglionically (N.M.), knee jerk (K.J.), and blood pressure (B.P.) in a cat (3.8 kg., chloralose anaesthesia). Records and numerals below as in Fig. 4. Responses before, during and after an infusion of 1,800 mg. of methylpentynol carbamate.

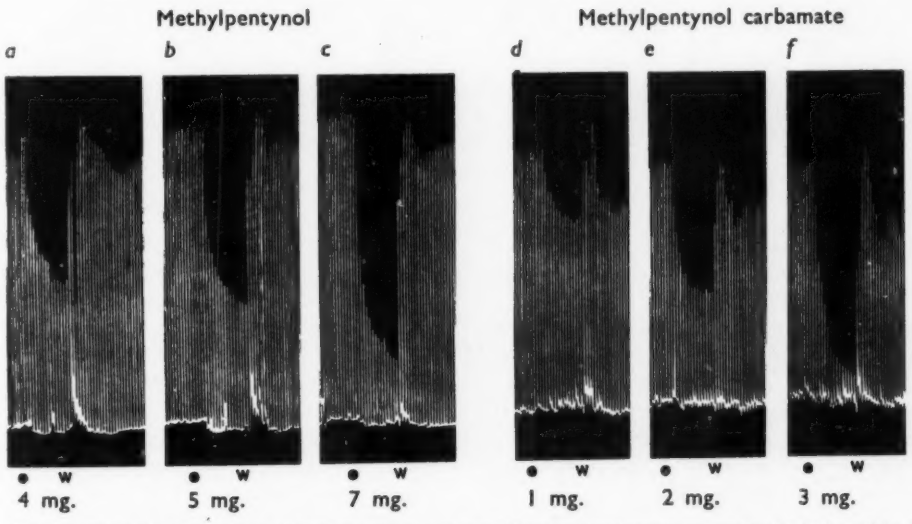

FIG. 6.—Effect of various doses of methylpentynol and methylpentynol carbamate on maximal twitches elicited by co-axial electrical stimulation of guinea-pig ileum (6/min.). Methylpentynol (*a*) 4 mg.; (*b*) 5 mg.; (*c*) 7 mg. Methylpentynol carbamate (*d*) 1 mg.; (*e*) 2 mg.; and (*f*) 3 mg. Bath vol. 10 ml. Contact time, 3 min. W indicates washing.

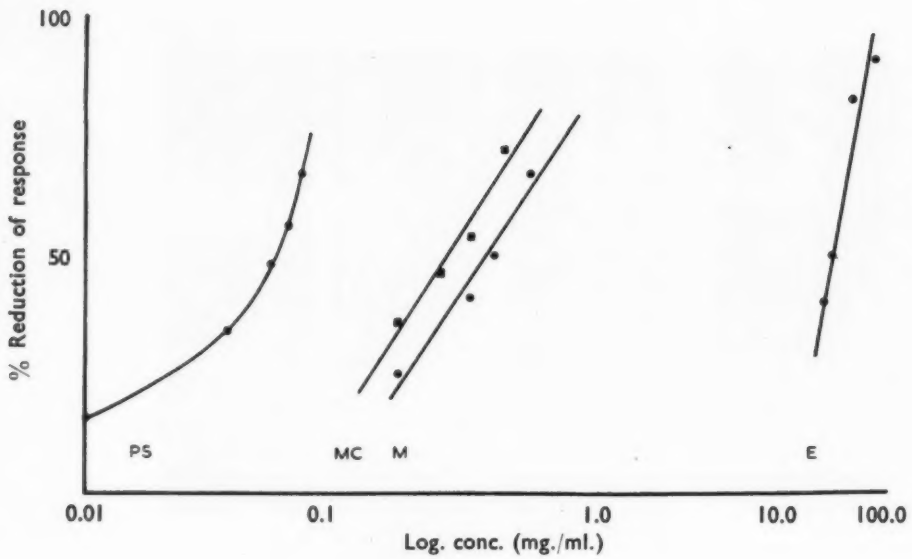

FIG. 7.—Dose/response relation for the reduction by pentobarbitone sodium (PS), methylpentynol carbamate (MC), methylpentynol (M), and ethanol (E) of the response of the guinea-pig ileum to acetylcholine.

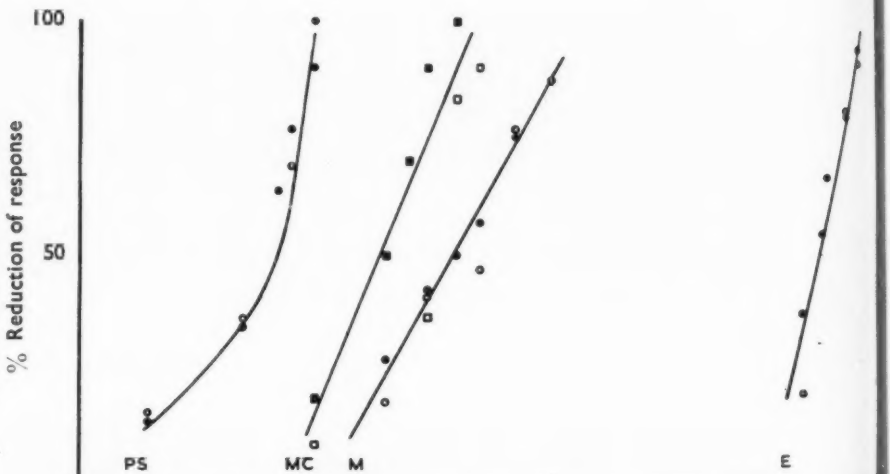

FIG. 8.—Dose/response relation for the reduction by pentobarbitone sodium (PS), methylpentynol carbamate (MC), methylpentynol (M), and ethanol (E) of the maximal twitch elicited by electrical stimulation of the guinea-pig ileum. Solid circles or squares indicate results from Expt. No. 1, open circles or squares those from Expt. No. 2.

produced only a small contracture. Ethanol in a solution approximately three times the molar strength of methylpentynol (4×10^{-3}) also had no effect. Both drugs in doses without apparent action on the rectus (Fig. 10*b*) potentiated the contractions due to submaximal doses of acetylcholine, an effect that persisted in the presence of either physostigmine (10^{-6}) or gallamine (5×10^{-6}) (Fig. 10*c*). The contraction elicited by decamethonium (5×10^{-6}) was also augmented.

Cat Sciatic Nerve Tibialis Anterior Muscle Preparation. — In 5 experiments, retrograde injections of up to 200 mg. (50 mg./kg.) of either methylpentynol or its carbamate into the contralateral common iliac artery had no effect on twitches elicited by single stimuli applied to the sciatic nerve tibialis anterior preparation. Similarly, no effect on this preparation was found with infusions of 2,884 mg. of methylpentynol or 1,627 mg. of methylpentynol carbamate, the respective drug blood concentrations immediately before death being 0.64 mg./ml. and 0.45 mg./ml. In the tetanized preparation, however, single intravenous injections of 50 mg./kg. (125 mg.) of methylpentynol carbamate or 100 mg./kg. (250

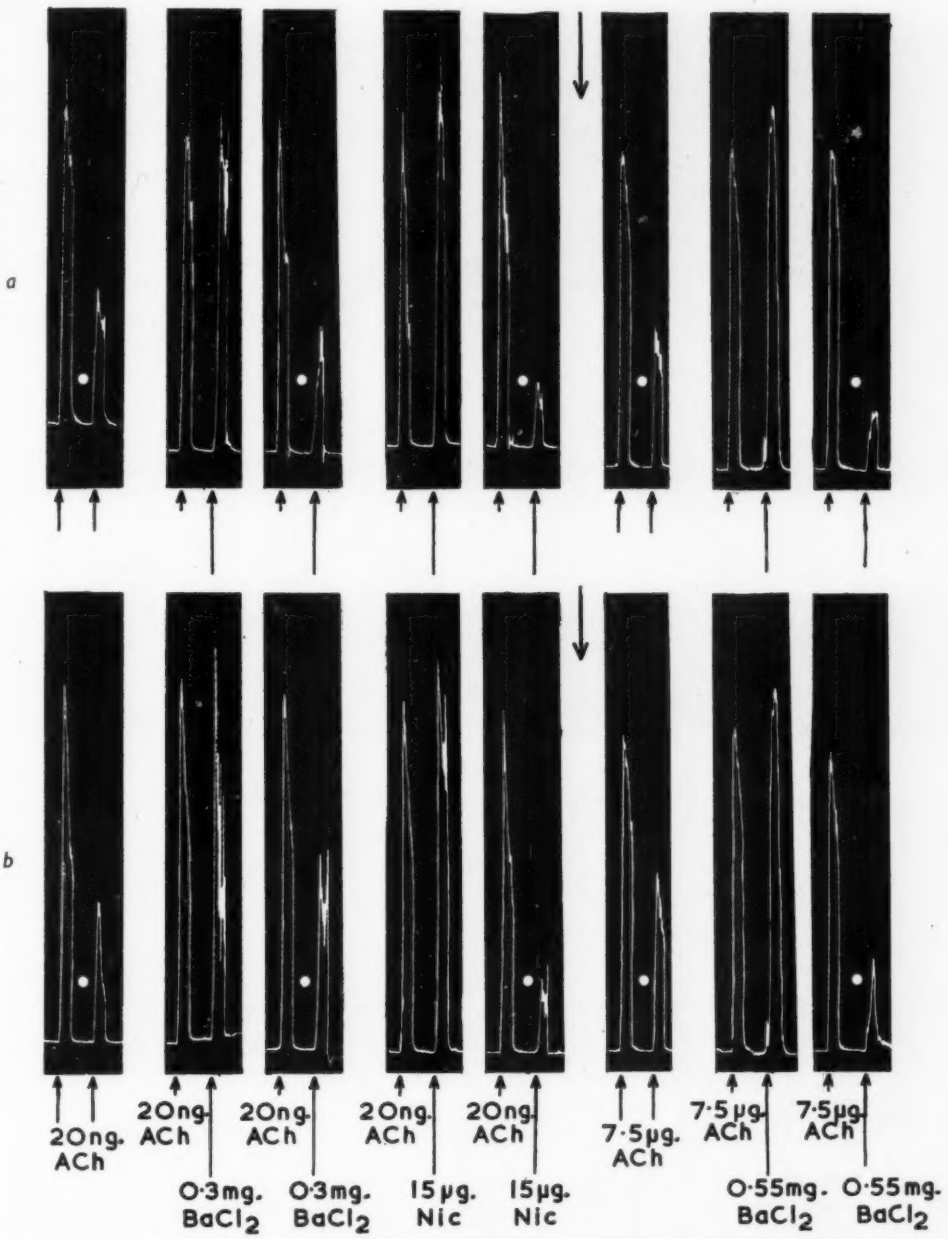

FIG. 9.—Isolated guinea-pig ileum preparation. Effect of 3.0 mg. of methylpentynol (at white dots in *a*) and of 1.75 mg. of methylpentynol carbamate (at white dots in *b*) on the half-maximal acetylcholine (ACh) contractions and matched $BaCl_2$ responses (before and after hyoscine 10^{-7} at downward pointing arrow) and matched nicotine (Nic) contractions. Note enhanced acetylcholine response after $BaCl_2$ and nicotine was allowed to return to normal before addition of methylpentynol or carbamate to bath.

mg.) of methylpentynol led to a 20% reduction of peak tension height followed by full recovery within 10 to 15 min. (Fig. 11). In the combined tibialis-soleus preparation, doses up to 100 mg./kg. (350 mg.) of methylpentynol carbamate or 250 mg./kg. (875 mg.) of methylpentynol had no effect on the single twitch response of these indirectly stimulated muscles, but with these doses artificial respiration was required.

Isolated Phrenic Nerve Rat Diaphragm Preparation. — In 2 experiments, diminution, preceded by brief augmentation of twitch, followed

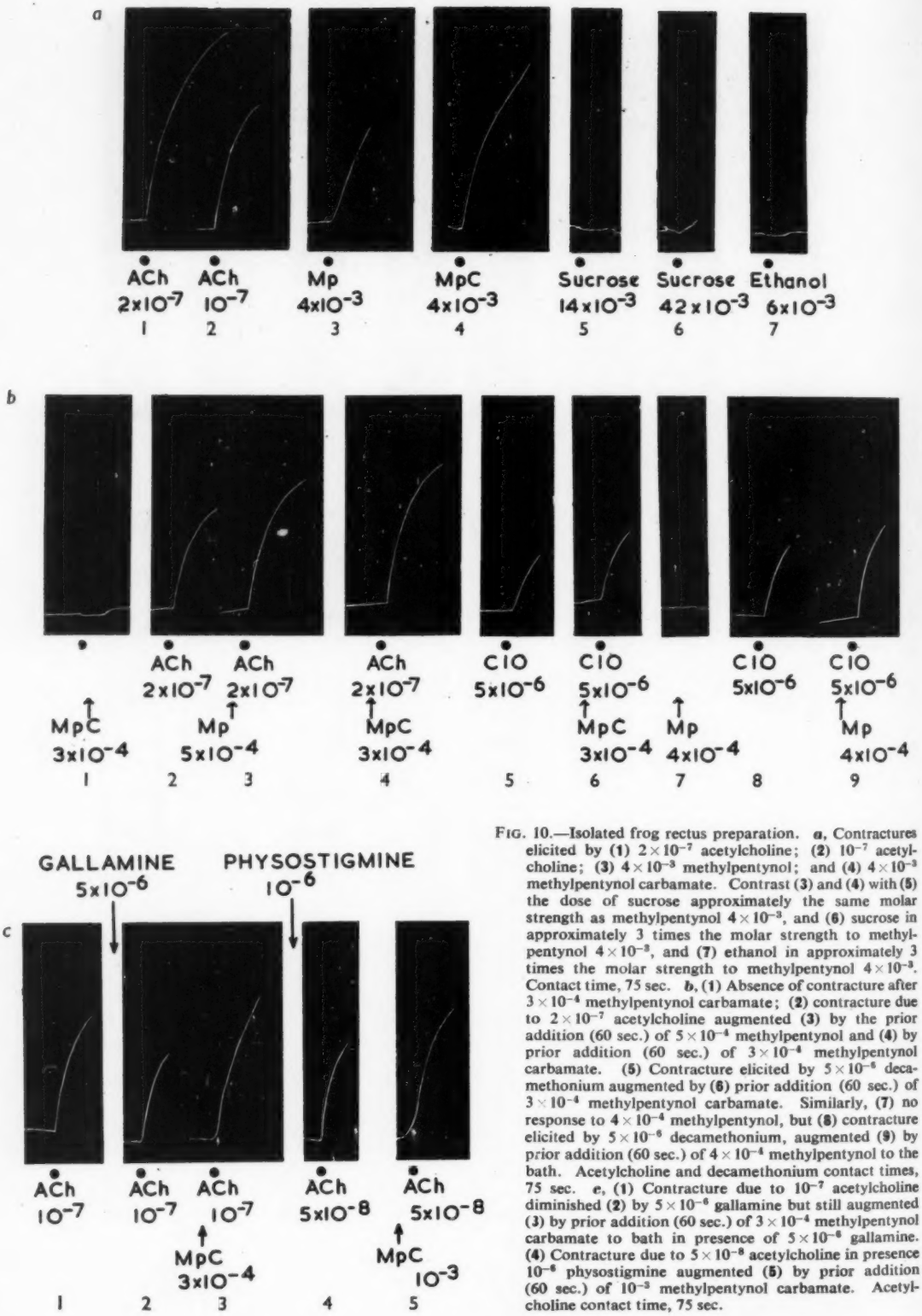

FIG. 10.—Isolated frog rectus preparation. *a*, Contractures elicited by (1) 2×10^{-7} acetylcholine; (2) 10^{-7} acetylcholine; (3) 4×10^{-3} methylpentynol; and (4) 4×10^{-3} methylpentynol carbamate. Contrast (3) and (4) with (5) the dose of sucrose approximately the same molar strength as methylpentynol 4×10^{-3}, and (6) sucrose in approximately 3 times the molar strength to methylpentynol 4×10^{-3}, and (7) ethanol in approximately 3 times the molar strength to methylpentynol 4×10^{-3}. Contact time, 75 sec. *b*, (1) Absence of contracture after 3×10^{-4} methylpentynol carbamate; (2) contracture due to 2×10^{-7} acetylcholine augmented (3) by the prior addition (60 sec.) of 5×10^{-4} methylpentynol and (4) by prior addition (60 sec.) of 3×10^{-4} methylpentynol carbamate. (5) Contracture elicited by 5×10^{-6} decamethonium augmented by (6) prior addition (60 sec.) of 3×10^{-4} methylpentynol carbamate. Similarly, (7) no response to 4×10^{-4} methylpentynol, but (8) contracture elicited by 5×10^{-6} decamethonium, augmented (9) by prior addition (60 sec.) of 4×10^{-4} methylpentynol to the bath. Acetylcholine and decamethonium contact times, 75 sec. *c*, (1) Contracture due to 10^{-7} acetylcholine diminished (2) by 5×10^{-6} gallamine but still augmented (3) by prior addition (60 sec.) of 3×10^{-4} methylpentynol carbamate to bath in presence of 5×10^{-6} gallamine. (4) Contracture due to 5×10^{-8} acetylcholine in presence 10^{-6} physostigmine augmented (5) by prior addition (60 sec.) of 10^{-3} methylpentynol carbamate. Acetylcholine contact time, 75 sec.

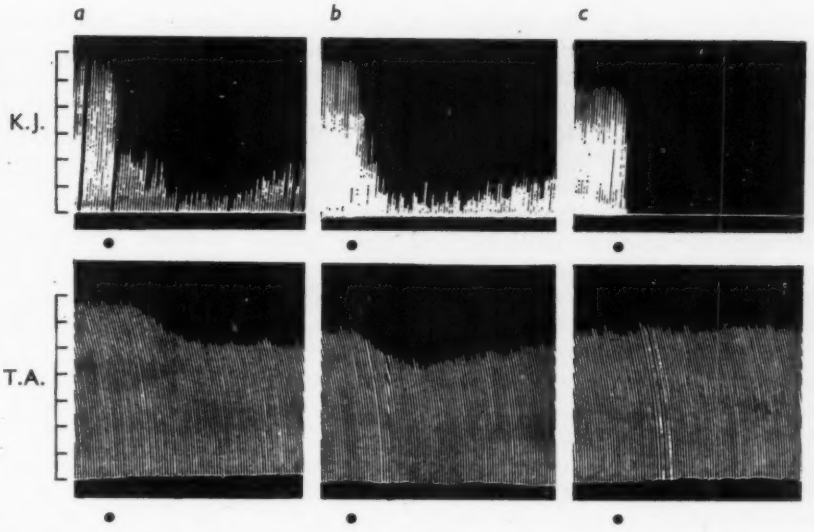

FIG. 11.—Records of knee jerk (K.J.) elicited at 12/min. and twitch of tibialis anterior muscle (T.A.) tetanized for 1 sec. in every 5 sec. in a cat (2.5 kg., anaesthetized with chloralose). Effect of single intravenous injections at black dots of (*a*) methylpentynol (100 mg.); (*b*) methylpentynol carbamate (50 mg.); and (*c*) sodium pentobarbitone (25 mg.). Scale, height of twitch in cm.

the addition to the bath of methylpentynol carbamate, methylpentynol and paraldehyde (M.W.132). After washing in Tyrode solution, the amplitude of the muscle twitch was restored to that seen in fresh muscle. Dose/response curves were plotted for the % reduction of maximum response 5 min. after the addition of the test substance to the bath (Fig. 12) and the approximate equipotent molar ratios were methylpentynol carbamate 1, methylpentynol 4.4, and paraldehyde 5.3.

Action on Gastric Secretion

In doses between 10 mg./kg. and 100 mg./kg. neither methylpentynol (5 experiments) nor its carbamate (2 experiments) had any constant action on histamine induced gastric secretion.

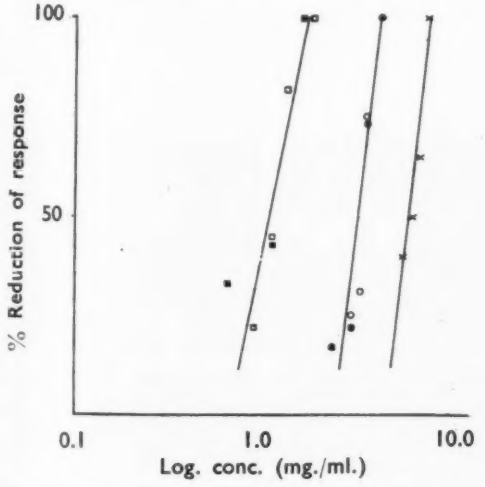

FIG. 12.—Dose/response relation for the reduction by methylpentynol carbamate (solid and open squares); methylpentynol (solid and open circles), and paraldehyde (crosses) of the maximal twitch of the indirectly stimulated rat phrenic nerve diaphragm preparation. Solid circles or squares are results from Expt. No. 1 and open circles or squares results from Expt. No. 2.

Action on the Nervous System

Anaesthesia. — The brain concentrations in guinea-pigs after intraperitoneal injections of methylpentynol during deep anaesthesia varied between 0.34 and 0.46 mg./g. and for the carbamate between 0.25 and 0.35 mg./g. (Table III). The righting reflex returned when brain concentrations of the drugs were still fairly high

TABLE III

BLOOD AND BRAIN CONCENTRATIONS OF METHYLPENTYNOL AND ITS CARBAMATE IN GUINEA-PIGS

Results from 12 male guinea-pigs following intraperitoneal injection of either methylpentynol or methylpentynol carbamate dissolved in 65% aqueous propylene glycol. The asterisk denotes result obtained from a total exsanguination sample. Brain concentrations of drugs are those for wet weight of organs.

Expt. No.	Weight (kg.)	Dose (mg./kg.)	Min. after Intraperitoneal Injection	State of Animal	Blood Conc. (mg./ml.)	Brain Conc. (mg./g.)
Methylpentynol						
1	0·552	400	15	Unconscious	0·15	0·34
2	0·510	400	20	,,	0·10	0·46
3	0·533	400	15	,,	0·06	0·37
4	0·446	300	270	Able to roll on side	0·03	0·21
5	0·565	300	100	,, ,,	0·06	0·17
6	0·546	400	125	,, ,,	0·21*	0·22
7	0·547	200	75	Righting reflex present	0·03	0·19
8	0·469	200	120	,, ,,	0·03	0·16
Methylpentynol carbamate						
9	0·469	200	15	Unconscious	0·15	0·35
10	0·525	200	15	,,	0·25	0·25
11	0·526	100	315	Righting reflex present	0·05	0·15
12	0·659	150	375	,, ,,	0·05	0·15

(0.16 to 0.19 mg./g. for methylpentynol and 0.15 mg./g. for methylpentynol carbamate). During anaesthesia, blood concentrations ranged between 0.06 mg./ml. and 0.15 mg./ml. with methylpentynol and 0.15 to 0.25 mg./ml. with its carbamate.

Similar results were obtained with cats anaesthetized first with ethyl chloride and ether, allowed to recover partially and then given intravenous injections or infusions of methylpentynol or the carbamate (3 experiments). A cat given a single intravenous injection of 250 mg./kg. of methylpentynol was still anaesthetized 20 min. later when the blood concentration of the drug was 0.33 mg./ml. Another animal infused with 20 mg. of methylpentynol/min. for 40 min. began to recover 15 min. after cessation of the infusion, by which time the blood concentration had fallen to 0.15 mg./ml. A cat given a single intravenous injection of 200 mg./kg. of methylpentynol carbamate was still anaesthetized 60 min. later with a blood concentration of 0.25 mg./ml.

Blood concentrations of methylpentynol in guinea-pigs after return of the righting reflex varied between 0.03 and 0.06 mg./ml., the one exception being Expt. No. 6 in which a high blood level was found, the specimen being obtained by total exsanguination. There was good accord between these values and results from unanaesthetized rabbits (2 experiments) which were given 100 mg./kg. daily of either methylpentynol or the carbamate by stomach tube, blood samples being removed from the ear vein before each dose of the drug. On the fourth day of this regime, the blood concentration of methylpentynol had risen to 0.03 mg./ml. and that of the carbamate to 0.035 mg./ml. and there were no signs referable to the central nervous system.

No free methylpentynol was found either in the blood or brain after the administration of methylpentynol carbamate.

Reflexes

Knee Jerk.—In 5 experiments, doses up to 200 mg. (100 mg./kg.) of ethanol were without effect on the knee jerk, but a 90% reduction in amplitude with full recovery in 50 min. followed the injection of single intravenous doses of 100 mg./kg. (250 mg.) of methylpentynol and 50 mg./kg. (125 mg.) of methylpentynol carbamate (Fig. 11). There was no augmentation before diminution of the knee jerk, maximum reduction occurring within 1 to 2 minutes of drug administration. More persistent diminution of the knee jerk was produced by intravenous infusion of the substances. Associated blood concentrations are shown in Figs. 4 and 5. Respiration tended to fail before disappearance of the knee jerk.

Reflex Alteration of Respiration.—The increase of respiratory minute volume in response to stimulation of the central end of the cut femoral nerve was reduced in 5 experiments for about 20 min. after a single intravenous injection of 100 mg. (30 mg./kg.) of methylpentynol and for 85 min. after the same dose of its carbamate (Fig. 13). The inhibition of respiration produced by stimulating the central end of the cut vagus remained unmodified in cats and rabbits after single intravenous injections of either 100 mg. (40 mg./kg.) of methylpentynol or of its carbamate.

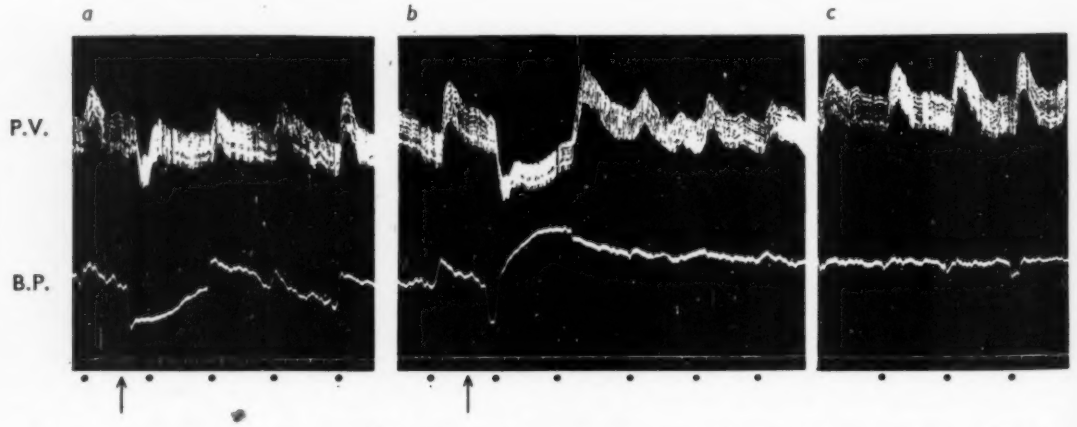

FIG. 13.—Record of pulmonary ventilation (P.V.) and blood pressure (B.P.) in a cat (3.3 kg., chloralose anaesthesia). Effect of intravenous injection at arrow in (*a*) of 100 mg. of methylpentynol; in (*b*) of 100 mg. of methylpentynol carbamate. In (*c*) recovery of response 85 min. after (*b*). Each black dot indicates stimulation of the femoral nerve at 10 sec. Time, min.

Ganglionic Transmission

Superior Cervical Ganglion (7 experiments).—The effect of intra-arterial injections of methylpentynol, methylpentynol carbamate and pentobarbitone sodium (their potencies in the reverse order) on the response of the nictitating membrane to preganglionic excitation is depicted in Fig. 14. A similar reduction of response occurred during the intravenous infusion of methylpentynol carbamate (Fig. 5). It transpired, during infusions of large quantities of methylpentynol or its carbamate, that the response of the nictitating membrane to direct stimulation by intravenous injections of 10 μg. of adrenaline was reduced *pari passu* with the decline in response to both pre- and post-ganglionic stimulation of the cervical sympathetic nerve. Once the contraction disappeared as a consequence of such infusions, it was impossible to elicit any response of the nictitating membrane either by adrenaline or pre- or post-ganglionic nerve stimulation for at least the next 6 hr. It was inferred that this was an effect of methylpentynol or carbamate on the muscle of the nictitating membrane, and not necessarily indicative of any action at the superior cervical ganglion.

To ascertain whether these drugs acted on the superior cervical ganglion, advantage was taken of the fact that both carotid arteries (from which the nictitating membrane and the superior cervical ganglion derive their blood supply) arise together from the innominate artery. Each drug was injected into the right internal mammary artery with the distal aorta occluded. That the injected substances reached both nictitating membranes in equivalent concentrations was indicated by identical responses to 10 μg. of adrenaline (Fig. 15). Injections of 25 mg. of methylpentynol carbamate and 50 mg. of methylpentynol led to similar evanescent relaxations of the nictitating membrane when stimulated preganglionically but not postganglionically, suggesting that the substances possessed weak ganglion-blocking properties, the carbamate being about twice as potent as methylpentynol.

Vasomotor Ganglia.—Pressor responses in 3 spinal cats which had received 1 mg./kg. atropine sulphate were induced by intravenous injections at

FIG. 14.—Record of contracture of the nictitating membrane in a cat (2.75 kg., chloralose anaesthesia) excited by continuous stimulation of cervical sympathetic at 10/sec. Single retrograde injections were made at black dots into the stump of the ligatured lingual artery, in (*a*) methylpentynol (12 mg.); in (*b*) methylpentynol carbamate (12 mg.); and in (*c*) sodium pentobarbitone, first 0.25 mg. and then 1 mg. Time, min. Scale, cm.

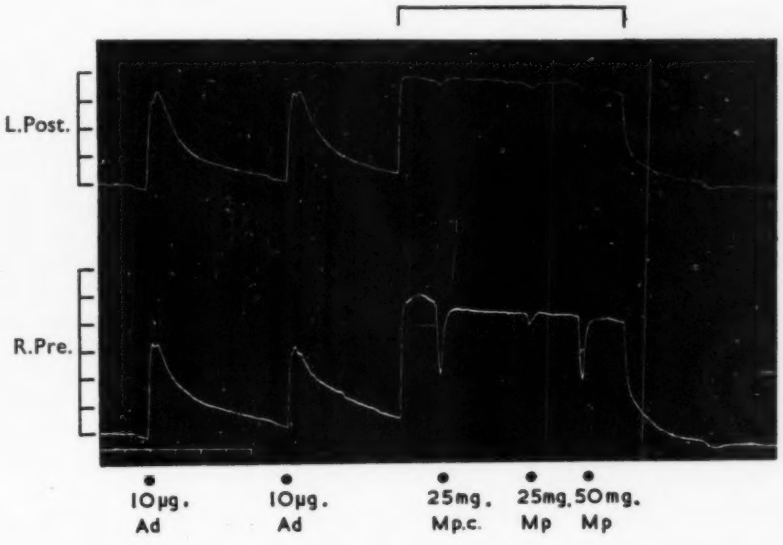

FIG. 15.—Record of contracture of both nictitating membranes in a cat (2.1 kg., anaesthetized with chloralose) excited by continuous preganglionic (R.Pre.) and postganglionic (L.Post) stimulation of cervical sympathetic at 10/sec. for period indicated. Single injections into right internal mammary artery of methylpentynol (Mp), of methylpentynol carbamate (Mp.c.) and of adrenaline (Ad). Time, min. Scale, cm.

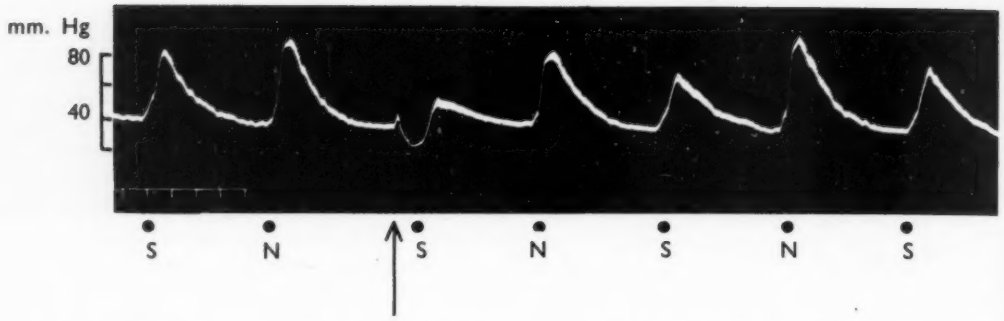

FIG. 16.—Effect on the blood pressure in a pithed and eviscerated cat (3.0 kg.) of intravenous injection of 150 mg. of methylpentynol (at arrow) 45 sec. before stimulation (10/sec.) of the peripheral end of the sectioned nerve to the left suprarenal gland (S) alternating every 5 min. with the intravenous injection of 3 μg. of noradrenaline (N). Time, min.

3 min. intervals of 0.4 mg. of nicotine alternating with 5 μg. of noradrenaline. Following the injection of 100 mg. of methylpentynol (50 mg./kg.) or of 50 mg. of methylpentynol carbamate (25 mg./kg.), the nicotine pressor response was reduced for 30 min. but that for noradrenaline remained unaltered.

Suprarenal Gland

Three pithed eviscerated cats were given injections of 3 μg. of noradrenaline alternating at 5 min. intervals with stimulation of the peripheral end of the sectioned nerve to the left suprarenal gland. Intravenous injections of 20 mg./kg. of methylpentynol (60 mg.) had no effect on the blood pressure response to suprarenal nerve stimulation, but a larger dose of 150 mg. of methylpentynol (50 mg./kg.) reduced the response (Fig. 16), full recovery occurring in about 20 min. The reaction to noradrenaline was little affected. The blood pressure response to stimulation of the sectioned nerve to the suprarenal gland 45 sec. after the injection of 60 mg. of methylpentynol carbamate (20 mg./kg.) was usually greater than the control; then, as with methylpentynol, the response to stimulation fell. Recovery was complete in 20 min.

DISCUSSION

It was intended to compare, as far as possible, the actions of methylpentynol and methylpentynol carbamate with those of other central nervous system depressants, particularly ethanol, and to correlate blood levels of the drugs with their activity. Marley and Vane (1958) likened the tissue and fluid distribution of both drugs following injection to that of ethanol, and suggested that the relation between methylpentynol and the carbamate resembles that between ethanol and urethane. But methylpentynol is a tertiary unsaturated alcohol, and as the activity of an alcohol increases with the size of its molecule (Gaddum, 1956) it was anticipated that methylpentynol would be more potent than ethanol.

Whereas ethanol in concentrations of 0.05 to 0.2% produces slowing of the heart with dysrhythmia and diminished systolic amplitude without effect on coronary flow in the perfused mammalian heart (Tunicliffe and Rosenheim, 1903; Backman, 1906), methylpentynol in concentrations of 0.1% is said not to affect rate of systolic contraction of either the isolated frog or rabbit hearts although a 50% increase of coronary flow ensued (Gialdroni and Grassi, 1952). In the present experiments with isolated kitten hearts, marked reduction of systolic amplitude and dysrhythmia together with a 15 to 30% increase of coronary flow occurred with the perfusion of solutions of 1:1,160 of methylpentynol or 1:750 of methylpentynol carbamate respectively.

Increases of arterial blood flow resulted from intra-arterial injections of either drug; and Hemmeter (1891) found augmented arterial blood flow after the intravenous injection of alcohol. The effect of alcohol on the blood pressure is a composite of its various actions on the heart, blood vessels and central nervous system. Both methylpentynol and the carbamate produced a fall of blood pressure in the intact animal, the carbamate being slightly more potent than methylpentynol. Smaller falls of blood pressure occurred in spinal and pithed cats.

There has been controversy as to whether alcohol stimulates respiration at low blood concentrations or not, although the stimulating action seems to have been demonstrated fairly conclusively (Wilmanns, 1897; Hitchcock, 1942), and seems to be due to a direct and not a reflex action. Certainly methylpentynol or its

carbamate, in single intravenous doses up to 10 mg./kg. in cats or as an infusion of 1.0 g. of methylpentynol in man, led to a small increase of respiratory rate and minute volume. Single intravenous doses greater than 20 mg./kg. resulted in short-lived respiratory depression in cats, both drugs being much less potent than pentobarbitone sodium. Margolin *et al.* (1951) stated that methylpentynol even in large doses did not depress respiration, although Roberts, Kane, Percival, Snow, and Please (1957) found that methylpentynol given to the pregnant mother depressed the respiratory minute volume of the newborn child. Both methylpentynol and the carbamate are more potent respiratory depressants than ethanol. According to Haggard, Greenberg, and Rakieten (1940) a blood concentration of 9.3 mg./ml. ethanol in fasting rats was associated with respiratory failure; this occurred in cats at a blood concentration of 1.2 mg./ml. with methylpentynol and 0.5 mg./ml. for the carbamate.

Both drugs reduced the response of the guinea-pig ileum to acetylcholine, $BaCl_2$, and nicotine. The contraction of the guinea-pig ileum to acetylcholine is mainly a direct effect on muscle, that produced by barium being mediated primarily through ganglia, whereas nicotine acts wholly through ganglia (Feldberg, 1951). Since both methylpentynol and the carbamate diminished the response of the guinea-pig ileum to acetylcholine and $BaCl_2$ in the presence of hyoscine 10^{-7}, their action is most likely to be on the smooth muscle. Histamine also contracts the guinea-pig ileum by a direct action on muscle, the response being attenuated by many central depressants including alcohols and urethanes (Farmer, 1938). That methylpentynol and the carbamate may also act on ganglia is shown by the fact that they produced greater diminution of the nicotine than of the acetylcholine and barium induced contractions of the isolated guinea-pig ileum. But the ganglionic action on gut is not great, since, in the cat, intravenous doses up to 60 mg./kg. of either drug or 240 mg./kg. ethanol in no way altered the contraction of the stomach *in situ* during vagal nerve stimulation. The results do not agree with those of Gialdroni and Grassi (1952), who found that, with the rabbit isolated intestine, methylpentynol had no "antispastic action with regard to the common convulsants (acetylcholine, histamine, barium)." The approximate equipotent molar ratios of methylpentynol and methylpentynol carbamate in reducing the response of the guinea-pig ileum stimulated electrically was 2.9:1, which agrees well with the ratio of 2.5:1 between LD50 values for intraperitoneal injection of the drugs into mice.

Methylpentynol and the carbamate in high doses elicited contractions of and augmented the submaximal acetylcholine contracture of the frog isolated rectus muscle, even in the presence of gallamine or physostigmine. Similarly, the decamethonium induced contracture of the rectus was increased. These phenomena could not be ascribed to changes in *p*H or osmotic effects of the drugs, and as neither substance dissociates into ions, an interaction with acetylcholine or similar receptors can hardly be postulated. Meng (1941) demonstrated that the sensitizing effect of alcohols and allied substances on the acetylcholine and nicotine contractions of the toad isolated rectus could not be related to inhibition of the cholinesterases but was non-specific and presumably attributable to their direct action on the muscle.

Both drugs have feeble neuromuscular blocking properties. They reduced the response of the indirectly stimulated phrenic nerve rat diaphragm preparation, the half-maximal response with methylpentynol occurring at a concentration of 2.7 mg./ml., well above that required for anaesthesia in cats. The twitch responses of the cat tibialis anterior or soleus muscle preparations in response to single stimuli applied to the sciatic nerve were unaffected by single intravenous injections of the drugs, and those of the tibialis anterior were unaltered even by infusions giving final blood concentrations of 0.64 mg./ml. of methylpentynol or of 0.45 mg./ml. of its carbamate. However, in the tetanized tibialis muscle preparation, small reductions of peak tension height followed the intravenous injections of 50 mg./kg. of the carbamate and 100 mg./kg. of methylpentynol. Quilliam (1955) noted that methylpentynol and paraldehyde produced neuromuscular block of the rat diaphragm preparation with unaltered response of the muscle to direct stimulation. Nicholls and Quilliam (1956) later concluded that the neuromuscular blocking activity of paraldehyde and methylpentynol could be accounted for only if they reduced the amount of acetylcholine released by nerve impulses.

The most striking effect of the drugs is on the central nervous system. Although anaesthesia has been said not to occur with "methylpentynol administered to dogs in sublethal amounts" (Margolin *et al.*, 1951), the experiments reported above show that unconsciousness could be produced by the intravenous injection of both drugs, persisting for at least 60 min. in one cat

given 200 mg./kg. of the carbamate. In guinea-pigs, intraperitoneal doses of 300 to 400 mg./kg. methylpentynol were followed by unconsciousness persisting for 1 to 4 hr., a similar result occurring with 100 to 150 mg./kg. of the carbamate. Brain concentrations of methylpentynol during deep anaesthesia varied between 0.34 and 0.45 mg./g. and between 0.25 and 0.35 mg./g. for the carbamate. As the wet weight of the brain is composed of about one tenth fat (McIlwain, 1955) and assuming this to behave like oleyl alcohol, then with a partition coefficient of 2.6 and a brain concentration of 0.35 mg./g. for methylpentynol, the molar concentration for anaesthesia would be 0.008 moles/l. Similarly for the carbamate, with a partition coefficient of 3.8 and a brain concentration of 0.25 mg./g., then the molar concentration for anaesthesia would be 0.005 moles/l.

The righting reflex of the guinea-pigs returned when brain concentrations of the drugs were still fairly high (0.16 to 0.19 mg./g. for methylpentynol and 0.15 mg./g. for the carbamate). This contrasts with the finding of Perlman and Johnson (1952) for rats given 800 mg./kg. of methylpentynol by gavage that no drug was found in any organ once the hypnotic effect had disappeared. The blood concentration on return of the righting reflex (some 75 to 375 min. after injection of the drugs) was 0.03 to 0.06 mg./ml. for methylpentynol or 0.05 mg./ml. for its carbamate. These blood and tissue results are consistent with the conclusion of Marley and Vane (1958) that the excretion and inactivation of either drug is slow. Their results were obtained from cats the majority of which were anaesthetized with chloralose. However, the cat has a much higher level of glucuronide excretion than other animals (Pryde and Williams, 1934), and as methylpentynol is eliminated as a conjugate of glucuronic acid (Perlman *et al.*, 1953) it could be argued that the persistence of methylpentynol or the carbamate for long periods in the cat was a consequence of their metabolic sparing, the available glucuronides having combined competitively with the chloralose. But the results from the guinea-pigs which received these drugs alone, from mice which often remained asleep 24 hr. after injection, and those from unanaesthetized rabbits in which blood concentrations of 0.030 to 0.035 mg./ml. were demonstrated for both drugs 24 hr. after their administration had ceased, show that prolonged action was not restricted to the cat under chloralose. Methylpentynol and its carbamate were shown also to persist in human beings for 48 hr. after withdrawal of these drugs (Bartholomew, Bourne, and Marley, 1958) in spite of the fact that in man (Perlman *et al.*, 1953) only minute amounts of methylpentynol are excreted as a glucuronic acid conjugate.

The failure to find any free methylpentynol in blood or brain after administration of the carbamate implies that these tissues cannot break down the carbamate, but the possibility still remains that after oral administration of the drug, acid hydrolysis occurs with release of small quantities of methylpentynol which is then absorbed from the stomach into the blood stream.

Jacobsen (1958) regarded methylpentynol as having no effect on polysynaptic reflexes. However, 30 mg./kg. doses of either drug produced quite long-lasting inhibition of the reflex increase of pulmonary ventilation following stimulation of the femoral nerve, although 40 mg./kg. doses of either drug failed to modify reflex inhibition of respiration produced by stimulating the central end of the cut vagus nerve. Single intravenous doses of 50 mg./kg. of the carbamate and 100 mg./kg. of methylpentynol significantly reduced the knee jerk which is mediated through a monosynaptic reflex and therefore should be fairly resistant to central nervous system depressants. During infusions with methylpentynol or the carbamate, reduction of the knee jerk only occurred with blood concentrations of the drugs known to be associated in cats and guinea-pigs with anaesthesia, respiration tending to fail before the knee jerk.

Quilliam (1957, 1959) obtained evidence that methylpentynol blocks transmission in the superior cervical ganglion of the cat and this was confirmed with both that substance and methylpentynol carbamate, the effect being transitory. During infusion experiments, a reduced response of the nictitating membrane to preganglionic excitation occurred only with blood concentrations of the drugs above those required for anaesthesia, but this is almost certainly due to an effect of the drug both on the membrane and on the ganglion. Suitable doses of methylpentynol or the carbamate also reduced the blood pressure response to stimulation of the peripheral end of the sectioned nerve to the left suprarenal gland, whereas the blood pressure response to injections of noradrenaline which acts directly on the blood vessels was little affected. Dicker and Steinberg (1957) found in man that 0.5 g. of methylpentynol by mouth diminished autonomic responses (increase in pulse rate or blood flow) provoked by anticipation of, or performance of, difficult tasks.

It is illuminating to contrast the almost identical results for equimolar potency ratios for the two drugs derived from a variety of whole animal and isolated tissue experiments (Table IV). The potency ratios of the drugs could well have been predicted for brain concentrations during anaesthesia (where the drug may well be distributed through a fat phase) from their equimolar potencies on the electrically stimulated guinea-pig ileum (where the drug is rapidly distributed through the Tyrode phase surrounding the tissue). That such a logical extension is possible furnishes more evidence for the consideration (Paton, 1957b) that the enteric autonomic network might be a valuable simple paradigm of the far more complicated networks of the brain itself.

TABLE IV

EQUIPOTENT MOLAR RATIOS OF METHYLPENTYNOL AND METHYLPENTYNOL CARBAMATE

The values are those obtained from experiments on whole animals or isolated organs.

	Methylpentynol	Methylpentynol Carbamate
LD50 (mice)	2·5	1
Guinea-pig brain conc. for anaesthesia (mole l.)	2·3	1
Reduction of response of nictitating membrane in the cat to preganglion stimulation ..	2·9	1
Reduction of knee jerk (cat) ..	2·9	1
ED50 (acetylcholine stimulated guinea-pig ileum)	2·1	1
ED50 (electrically stimulated guinea-pig ileum)	2·9	1
ED50 (rat diaphragm phrenic nerve prep.)	4·4	1

Thus while the distribution of methylpentynol and its carbamate in the tissues is more reminiscent of that for ethanol than that of the barbiturates, their activity resembles both types of drug but with a different order of potency. On the electrically stimulated guinea-pig ileum, methylpentynol is 77 times more potent than ethanol but approximately 14 times less so than pentobarbitone sodium, the blood concentrations associated with unconsciousness being 10 to 20 smaller than those for ethanol. Even the features of intoxication in man produced by methylpentynol or methylpentynol carbamate closely resemble that seen with alcohol or barbiturates (Marley and Bartholomew, 1958). It would be unwise therefore to stigmatize methylpentynol as only another alcohol (or the carbamate as merely its ester). Both substances exhibit a number of distinctive properties in animals and humans.

This work was carried out during the tenure of a Medical Research Council Clinical Research Fellowship. I am indebted to Professor W. D. M. Paton and Dr. J. R. Vane for their considerable advice and encouragement. Thanks are due also to Dr. A. G. Mezey for his help with the recording of expiratory volume in man, to Dr. J. B. E. Baker for the loan of his perfusion apparatus, and to British Schering Ltd. for a generous supply of methylpentynol and methylpentynol carbamate. I should like also to thank Mr. D. A. Green and his staff for technical assistance.

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THE EFFECT OF METHYLPENTYNOL AND METHYLPENTYNOL CARBAMATE ON THE PERFUSED SUPERIOR CERVICAL GANGLION OF THE CAT

BY

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The action of methylpentynol and methylpentynol carbamate on the perfused superior cervical ganglion of the cat was analysed. Both drugs, in doses of 1 to 5 mg., depressed the output of acetylcholine. If the acetylcholine output was reduced by more than about 50%, transmission failed. Both drugs also reduced the response of the ganglion to injected acetylcholine. The carbamate, but not methylpentynol itself, had a transient stimulant action on the ganglion, unaccompanied by acetylcholine release.

Quilliam (1957, 1959) has obtained evidence that methylpentynol blocks transmission in the superior cervical ganglion of the cat. This has been confirmed for both methylpentynol and its carbamate (Marley, 1959), although the effect on the blood-bathed ganglion was evanescent. It remained, however, to determine whether these drugs have specific ganglion-blocking properties or depress ganglionic function in a less specific way, as do, for instance, the barbiturates (Exley, 1954).

METHODS

Cats anaesthetized with chloralose (80 mg./kg.) were used. Contractions of the nictitating membrane (after enucleation of the eyeball) were evoked by application of maximal shocks of 0.5 msec. duration at 10 c./s. to the cut peripheral stump of the cervical sympathetic, divided and separated from the vagus in the neck. The contractions were recorded on smoked paper by an isotonic lever with 4.0 g. load and seven-fold magnification. The superior cervical ganglion was perfused by the method of Kibjakow (1933) and Feldberg and Gaddum (1934), the perfusion fluid being warmed to the temperature of the cat by its passage through a polythene tube inserted into the stomach of the animal and which emerged from a small cut in the oesophagus close to the ganglion (Emmelin and Macintosh, 1956). The perfusion fluid was Locke solution, also containing eserine (5×10^{-6}) when acetylcholine output was to be measured. When the perfusion had been established and the last trace of blood washed from the system, samples of the effluent were collected usually for consecutive 6 min. periods, or for longer times if flow was slow. Preganglionic stimulation, when used, was applied only for the first 3 or 4 min. of these periods, the remaining interval being allowed for washing out the acetylcholine released during the stimulation by a sufficient volume of perfusate. The acetylcholine in the perfusate was assayed against acetylcholine chloride on a strip of guinea-pig ileum treated with 5 μg./l. of neostigmine and 10 mg./l. of morphine (Paton, 1957). That the substance in the perfusate released by stimulation of the preganglionic sympathetic was acetylcholine was verified first by showing that the contractor effect on the guinea-pig ileum was lost after the addition of hyoscine (10^{-7}) to the bath, and second by making a sample alkaline and standing it at room temperature for 20 min., neutralizing, then reassaying to demonstrate absence of contractor activity. Activity did not appear unless eserine had been added in adequate proportions to the perfusion fluid. The outputs shown in Figs. 3 and 4 are expressed ng. in unit time, so that the area of each rectangle gives the total output during each period.

For intra-arterial injection, pure methylpentynol was dissolved in Locke solution (20 mg./ml.). A similar concentration of methylpentynol carbamate could be dissolved in Locke solution if the solution was gently warmed and then allowed to cool. Acetylcholine chloride prepared in 0.9% saline was used for assay or injection.

RESULTS

Action on Perfused Superior Cervical Ganglion of the Cat

Both methylpentynol and its carbamate (in intra-arterial doses of 25 to 50 mg.) act at the blood-bathed ganglion to produce a relaxation of the nictitating membrane contracted by continuous preganglionic stimulation of the cervical sympathetic nerve, while the post-

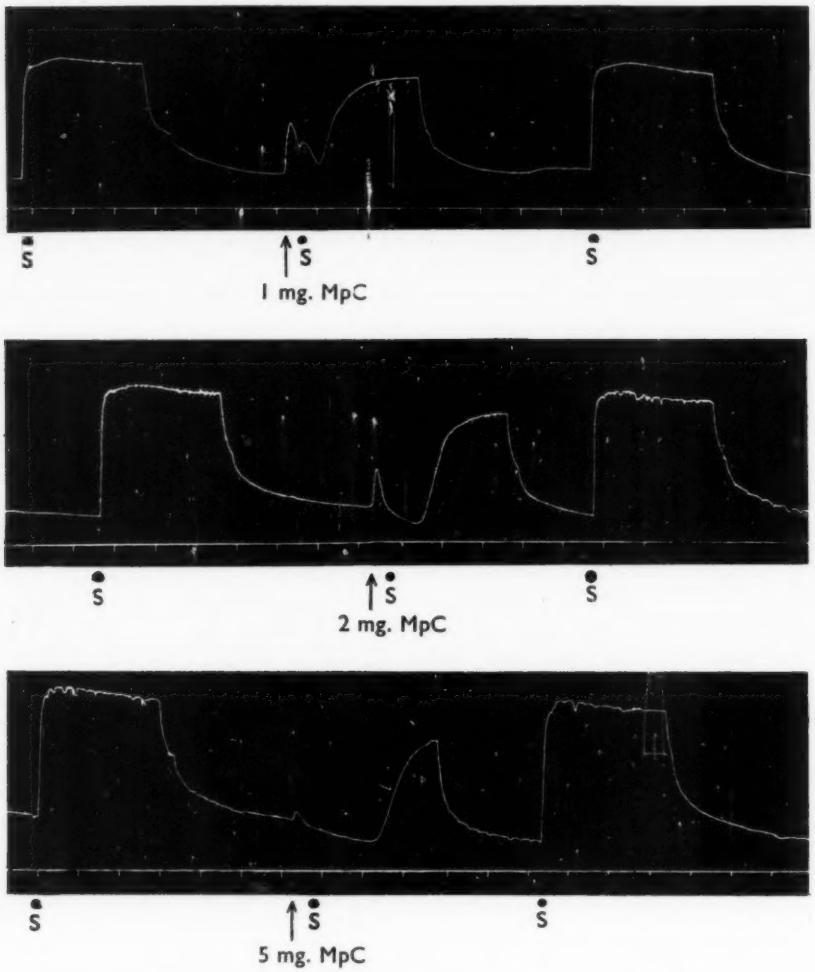

FIG. 1.—Cat, 1.7 kg.; chloralose anaesthesia. Perfused superior cervical ganglion preparation. Effect of various doses of methylpentynol carbamate (MpC) given intra-arterially on the contracture of the nictitating membrane excited by stimulation of the cervical sympathetic sustained for 3 min. at 10/sec. (at S). Note also small contraction elicited by methylpentynol carbamate. Time, min.

ganglionic response is preserved (Marley, 1959) but the response of the intermittently stimulated nictitating membrane was only reduced by the intravenous infusion of very large doses (1,800 mg.) of either drug. With the perfused ganglion, likewise relatively large doses (1 to 5 mg.) were needed. In this preparation, injections into the perfusion cannula of the carbamate, but not of methylpentynol itself, sometimes produced a brief contraction of the nictitating membrane before the ganglionic block appeared (Fig. 1). This was never seen with injections to the blood-bathed ganglion, nor did the carbamate ever enhance the contraction produced by electrical stimulation of the cervical sympathetic. The contraction produced by the carbamate was not accompanied by release of acetylcholine in the perfusate.

If the ganglion was stimulated by acetylcholine injected into the perfusion cannula (eserine being absent from the perfusing fluid), the response of the nictitating membrane was transiently diminished by previous doses of 2.0 mg. methylpentynol or the carbamate into the perfusate, although the response to preganglionic stimulation was unaffected (Fig. 2). The response to injected acetylcholine was thus more readily antagonized than that in normal transmission. The acetylcholine response usually returned to normal within 3 to 6 min. after giving either drug. If the preganglionic response was reduced by higher

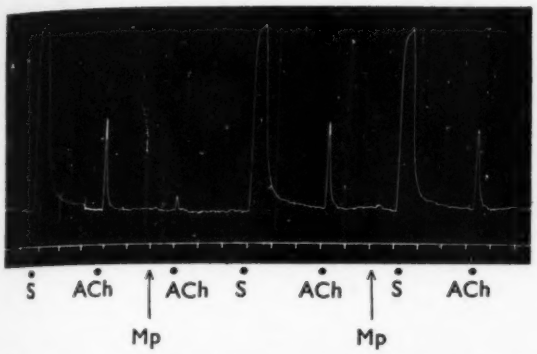

FIG. 2.—Cat, 5.3 kg.; chloralose anaesthesia. Perfused superior cervical ganglion preparation. Effect of 2.0 mg. of methylpentynol (Mp) on the response of the nictitating membrane to injections of 50 μg. of acetylcholine (ACh) or stimulation (S) of the cervical sympathetic 10/sec. for 1 min. Intra-arterial injections.

doses of methylpentynol or the carbamate, the effect was generally of greater duration than with the blood-bathed ganglion.

Effect on Release of Acetylcholine

Five experiments were made. In three, 1 mg. of methylpentynol or the carbamate reduced acetylcholine output during stimulation, although the mechanical response by the nictitating membrane was not necessarily affected. Thus in one experiment, after 1.0 mg. of methylpentynol, the response of the nictitating membrane declined, and the acetylcholine content of the effluent fell from 25 ng./ml. (total, 100 ng.; stimulation period, 4 min.) to 12 ng./ml. (total, 28 ng.). In another preparation after 1.0 mg. of the carbamate (the second injection seen in Fig. 3), the nictitating membrane response was unaltered, although the acetylcholine output fell from 16.2 ng./ml. (total, 27.5 ng.; stimulating period, 3 min.) to 8.3 ng./ml. (total, 13.4 ng.). The action of these doses was sometimes transient; thus, in the experiment in Fig. 3, the first injection of 1 mg. of the carbamate, given 6 min. before stimulation, failed to have any effect. On the other hand, in the same experiment, a larger dose (5 mg.) had a prolonged action; the output fell immediately from 17.5 ng./ml. (total, 24.2 ng.; stimulation period, 3 min.) to 3.7 ng./ml. (total, 3.9 ng.), followed by a partial recovery (10.6 ng./ml.; total 12.8 ng.) during which the nictitating response returned to normal. Sometimes even 1 mg. of either drug had a prolonged action; in one such experiment injections of 3 μg. and 2.5 mg. of choline in successive periods failed to relieve the reduction of acetylcholine output.

In one experiment, 10 mg. of methylpentynol was injected. The response of the nictitating membrane disappeared, but the total quantity of acetylcholine in the immediate stimulated specimen rose from 8 ng./ml. (total, 38.4 ng. in 14 min.) to 37 ng./ml. (total 66.6 ng. in 14 min.) but declined thereafterwards (Fig. 4). That this augmented acetylcholine output may have been merely the result of a damaging action of the drug on the ganglion, releasing substances usually bound, is suggested from another experiment in which a gut-relaxing substance appeared in the perfusate (in the absence of eserine) after the injection of the same amount of methylpentynol. The relaxation of the guinea-pig ileum observed was too great to have been due to methylpentynol itself, and the relaxant substance did not diminish the acetylcholine contractions of the rat uterus. The observation was not pursued, since block could be produced by smaller doses without these additional effects.

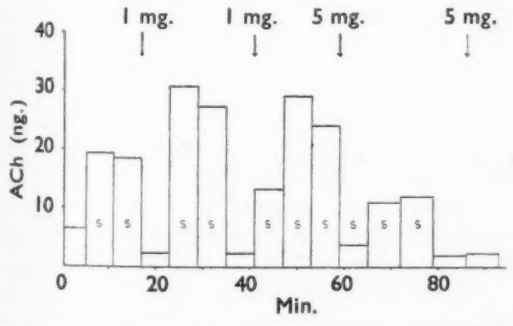

FIG. 3.—Histogram of acetylcholine output from a perfused superior cervical ganglion of the cat. Effect of various doses of methylpentynol carbamate (doses in mg. intra-arterially at arrows) on output of acetylcholine (ACh) in 6 min. periods; stimulation of cervical sympathetic for 3 min. during periods marked S.

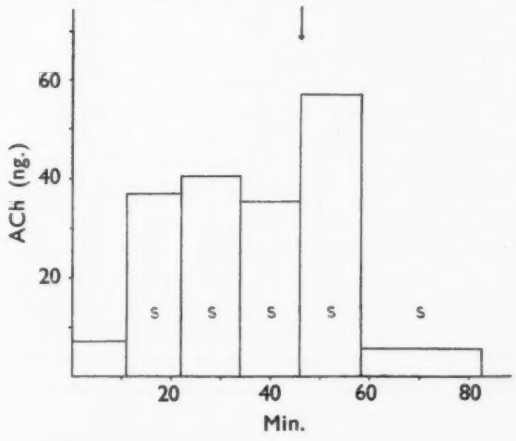

FIG. 4.—Histogram of acetylcholine output from a perfused superior cervical ganglion. Effect of 10 mg. of methylpentynol (intra-arterially at arrow) on output of acetylcholine (ACh) in 12 min. periods; stimulation of cervical sympathetic for 3 min. during periods marked S.

Methylpentynol or the carbamate in concentrations of 0.3 to 0.5 mg./ml. in the assay bath were found to antagonize acetylcholine about 2.3-fold: methylpentynol or its carbamate was therefore added to the standard acetylcholine solutions during the assays of samples of the perfusate following injections of these drugs.

Discussion

Nicholls and Quilliam (1956) concluded that the neuromuscular blocking activity of methylpentynol could be accounted for only if it reduced the amount of acetylcholine released by nerve impulses. They had shown that neuromuscular blocking concentrations of methylpentynol with a frog nerve-muscle preparation do not block transmission in the nerve, do not depolarize the end plate region, do not act like tubocurarine, and do not decrease the excitability of the muscle membrane. In our experiments, it was shown that these drugs do indeed depress acetylcholine release.

Methylpentynol does not, however, reduce acetylcholine output very powerfully. Transmission failed when the output had been reduced by roughly 50% or more; but it is known that, during continued excitation, outputs considerably lower than this are compatible with almost normal transmission (Brown and Feldberg, 1936). Failure of transmission must also depend, therefore, on the reduction by methylpentynol of the response of the ganglion cell to acetylcholine.

The excitant action of the carbamate is of interest, since it has also been found that, in the intact animal, the pressor response to splanchnic nerve stimulation increased before a subsequent diminution, after methylpentynol carbamate was given, but not after methylpentynol itself (Marley, 1959). The two observations suggest that the carbamate moiety confers the ability to excite ganglia. Further, since this excitation in the perfused ganglion dwindled as the dose increased, it is probably antagonized by the dominant blocking action of the molecule. The known instability of the carbamate ion in solution, rapidly forming ammonium carbonate, probably excludes an action by carbamate released from its esterification with methylpentynol. If this is the case, then the stimulant action must be attributed to the introduction of the basic carbamino group into an otherwise depressant molecule.

The combination of reduced acetylcholine output and antagonism to injected acetylcholine, as a result of injection of these drugs into the ganglion, suggests that they should be regarded as general ganglion depressants. Specific ganglion blocking agents such as curarine, nicotine, the methonium salts, and pempidine produce ganglion block without interfering with release of acetylcholine (Feldberg and Gaddum, 1934; Feldberg and Vartiainen, 1934; Paton and Zaimis, 1951; Corne and Edge, 1958). The properties displayed by methylpentynol, however, correspond more closely to those of procaine (Harvey, 1939), procaine amide (Paton and Thompson, 1953) and the barbiturates (Exley, 1954). It may well be the case that any drug capable of depressing the activity of nervous tissue in some way will as a general rule reveal this action at the ganglionic synapse too, presynaptically by interfering with normal release of the transmitter, and postsynaptically by desensitizing the recipient neurone to its action.

This work was carried out during the tenure by one of us (E. M.) of a Medical Research Council Clinical Research Fellowship. We are indebted to British Schering Ltd. for a generous supply of methylpentynol and methylpentynol carbamate, and to Mr. D. A. Green and his staff for technical assistance.

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THE EFFECT OF ADRENAL STEROIDS AND CORTICOTROPHIN ON THE GROWTH OF A SARCOMA OF HUMAN ORIGIN IN SMALL LABORATORY ANIMALS

BY

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(RECEIVED JANUARY 8, 1959)

9α Fluorohydrocortisone, prednisolone, and hydrocortisone promoted the growth of the human tumour (H.S.1) in weanling rats, but deoxycortone acetate was ineffective even when injected in very high dosage; Cortrophin ZN, a long-acting corticotrophin preparation, was just as effective as cortisone. This effect of corticotrophin on tumour growth was mediated by the adrenal cortex, and was related to the enhanced secretion of adrenocorticoids; some correlation existed between tumour size and adrenal hypertrophy in the corticotrophin-treated rats. The action of adrenal steroids and of corticotrophin in promoting the growth of the tumour is discussed.

Very few human tumours will proliferate well enough in animal hosts to enable chemotherapeutic and other cancer studies to be performed. However, it has been reported (Toolan, 1951a, 1951b, 1952, 1953a, 1953b, 1954; Toolan and Moore, 1952; Chute, Sommers, and Warren, 1952; Sommers, Chute, and Warren, 1952; Patterson, Chute, and Sommers, 1954; Towbin, 1954; Handler, 1956; Handler, Davis, and Sommers, 1956) that human tissues, both normal and malignant, can be grown in small laboratory animals, providing these hosts are treated by X-irradiation and/or cortisone. Such treatment is essential to reduce the strong natural resistance of the animals to heterografts. Hamsters, however, appear to show much less resistance than any other animal, and tumour growth can occur in untreated newborn hamsters (Chesterman, personal communication).

The tumour H.S.1 was obtained by Toolan in January, 1953, as a soft white mass; a cube of approximately 1 cm. in size, from the calf of the leg of a 43-year-old male, and was one of the few to be successfully passaged in animal hosts. Although fast growing, this tumour has never been grown subcutaneously or intraperitoneally in animals untreated by X-irradiation or by cortisone, except in newborn hamsters (Chesterman, personal communication). Toolan (1954) has confirmed that this tumour contains human antigens when tested by the agar plate method of Ouchterlony (1953).

The present investigation was commenced to extend the studies of Toolan, and in particular to investigate the effect of other adrenocortical steroids, and of corticotrophin on the growth and development of the tumour H.S.1.

METHODS AND MATERIALS

All animals were maintained on a No. 41b cube diet and tap water to which 0.01% oxytetracycline (Terramycin) was added to reduce the incidence of infection. Preliminary investigations showed the importance of using weanling rats; male Wistar rats of the A.R.C. strain of body weights 30 to 35 g. (18 to 21 days old) were found to be the most suitable; if slightly older and larger animals were used the tumours were poor in growth and development or alternatively failed to grow at all. In early experiments, the rats were treated with both X-irradiation and cortisone; they received 150 r total body X-irradiation 1 to 3 days prior to the tumour implant, and 4 subcutaneous injections of cortisone acetate (3 mg./rat). The steroid was injected into the area of the left shoulder, as far away from the site of tumour implant as possible to eliminate any chance of local action of the hormone on tumour growth. In later experiments rats were not X-irradiated but received the cortisone treatment alone. The steroid was injected in 4 subcutaneous doses each of 20 mg./100 g. body weight; the first injection was given at the time of tumour implant, the others at 48 hr. intervals.

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Hamsters received a single subcutaneous injection of cortisone acetate (20 mg./100 g. body weight) at the time of implant, and no further treatment was given.

Weanling albino mice of the A.R.C. strain of body weights 8 to 11 g. were used in a few experiments; these animals received 3 subcutaneous injections of cortisone acetate into the left shoulder area. The first dose (3 mg./mouse) was given at the time of the tumour implant, the remaining injections (2 mg./mouse) were given at 48 hr. intervals.

Tumours were removed aseptically and minced in a sterile tube. A suspension of the tumour tissue was made in a sterile Ringer fluid (NaCl 0.9%, KCl 0.042%, $CaCl_2$ 0.024%, $NaHCO_3$ 0.015%) containing glucose (1.0%), penicillin (200 units/ml.) and streptomycin (200 μg./ml.). Approximately 4 ml. of this solution was added to each gram of tumour tissue. Each rat received 0.5 ml. of tumour suspension injected subcutaneously into the shaved right flank using a No. 16 or 18 needle. Mice received 0.25 ml. of the suspension in a similar site, while hamsters were lightly anaesthetized with sodium pentobarbitone (Nembutal, 9 mg./100 g.) intraperitoneally and were injected with 0.25 ml. of the tumour suspension into one or both of the cheek pouches, or 0.5 ml. into the flank (Handler, 1958). In all hosts, the tumour was harvested on or about the fourteenth day after implantation.

Results

Choice of Host Animal.—The tumour was successfully grown in cortisone treated rats, hamsters, and mice; the rats were the animals of choice for general investigations. In all the experiments reported here, the animals were treated with cortisone or other adrenal steroid alone; X-irradiation in combination with cortisone was only used in the initial experiments on the passage of the tumour. Since there appeared to be no difference between the rate of growth of the tumour in rats receiving cortisone alone and in those treated with a combination of X-irradiation and cortisone, the former treatment was selected. Fig. 1 shows the appearance of the tumour in a rat 14 days after implantation, the implant having been made subcutaneously into the right flank.

Toolan (1954) reported that with the strain of rats used in her experiments, the tumour killed the host within 12 to 15 days from implantation, the animal dying in a cachectic and emaciated condition. In the present series of experiments this mortality was seen in some of the animals, especially in those bearing large tumours which sometimes grew to 25% of their body weight; in other rats there was a gradual necrosis and regression of the tumour with no apparent ill effect on the animal host. This regression of the tumour was also seen when rats were treated with corticotrophin instead of cortisone.

Hamsters were used solely to provide a reserve supply of tumour tissue for routine passage; mice were used in a few experiments, but they had no distinct advantage over rats; moreover, in many instances mice showed a marked toxic reaction to the cortisone treatment (D'Arcy and Howard, 1958).

The tumour was successfully passaged within and between the three species of host, namely from rat to rat, from rat to hamster cheek pouch, and from rat to mouse and *vice versa*. Some difficulty was occasionally experienced in mouse to mouse passage; the reason for this was not fully evident, but it is believed to be due, at least in part, to the poor condition of the mice receiving large doses of cortical steroids.

The Effects of Some Adrenal Steroids on Tumour Growth.—The effects of some of the more recent analogues of cortisone and of hydrocortisone on the growth and development of the sarcoma in the rat were investigated. The results of experiments with cortisone acetate, hydrocortisone acetate, prednisolone, 9α fluorohydrocortisone acetate, and deoxycortone acetate are summarized in Table I. The doses of steroid were injected subcutaneously in saline suspension immediately after tumour implantation and were repeated three times at 48 hr. intervals.

TABLE I

THE EFFECT OF SOME ADRENAL STEROIDS ON THE GROWTH OF SARCOMA H.S.1 IN WEANLING RATS

The steroids were administered four times subcutaneously on alternate days.

Steroid (mg./100 g. Body Wt.)	Results
Cortisone acetate:	
80	Toxic
40	"
20	Growth
10	"
5	Slight growth
Hydrocortisone acetate:	
20	Growth
10	"
Prednisolone (Δ^1 hydrocortisone):	
5	Growth
2·5	No growth
9α Fluorohydrocortisone acetate:	
4	Toxic
2	Growth
1	"
0·5	Slight growth
Deoxycortone acetate:	
200	No growth
100	" "
20	" "

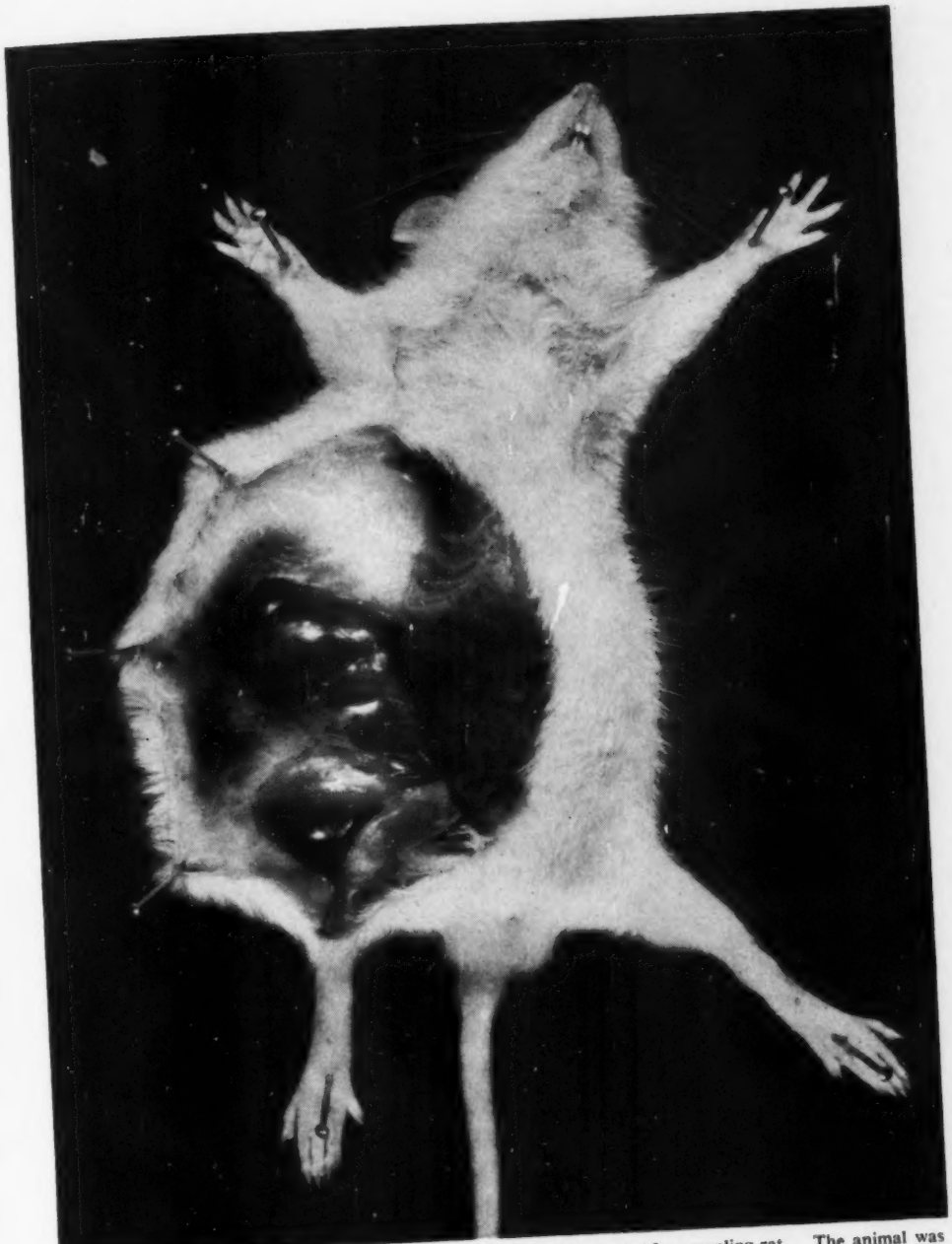

FIG. 1.—Sarcoma H.S.1; 14 days after implantation in the right flank of a weanling rat. The animal was given 20 mg. of cortisone acetate/100 g. body weight subcutaneously at the time of implantation of the tumour and 3 further doses at 48 hr. intervals.

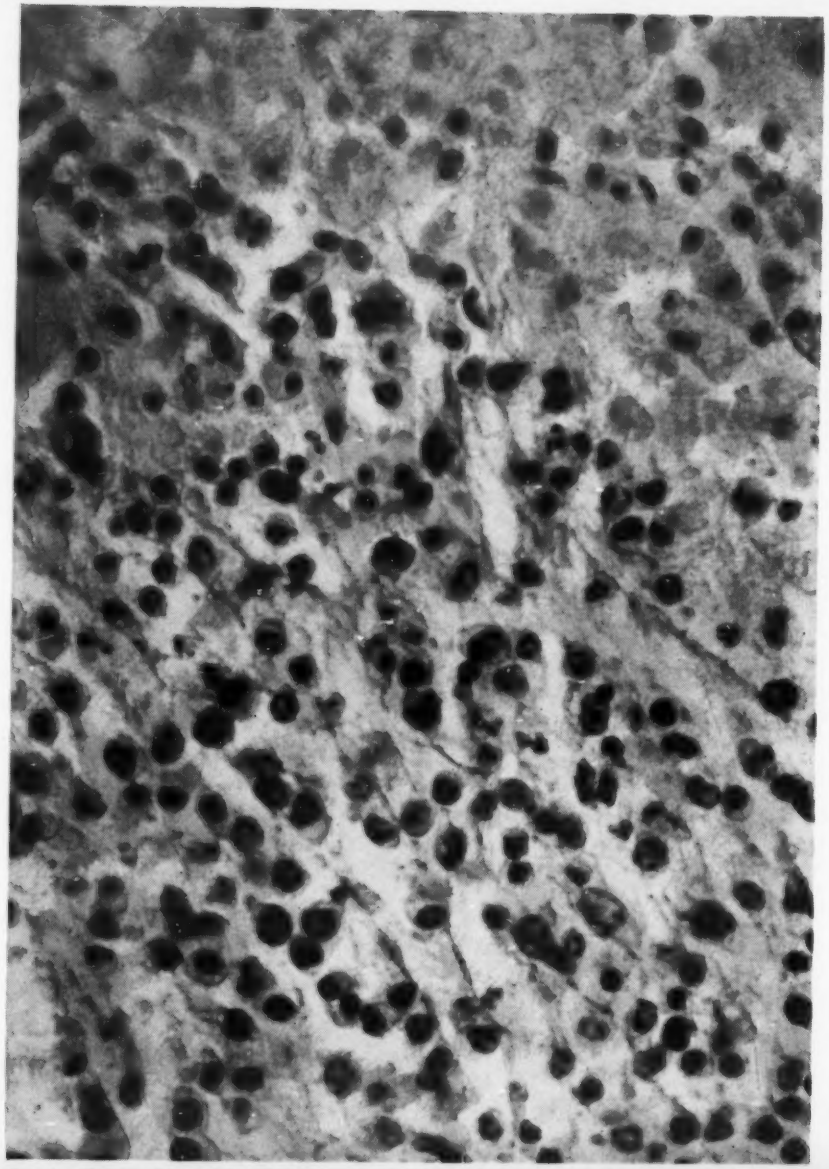

FIG. 2.—Sarcoma H.S.1, 14 days after implantation into the flank of a weanling rat treated with cortisone acetate, 20 mg./100 g. body weight subcutaneously at the time of implant and 3 further doses at 48 hr. intervals. A multinucleated round cell sarcoma with extensive fibrous elements. Magnification, × 350.

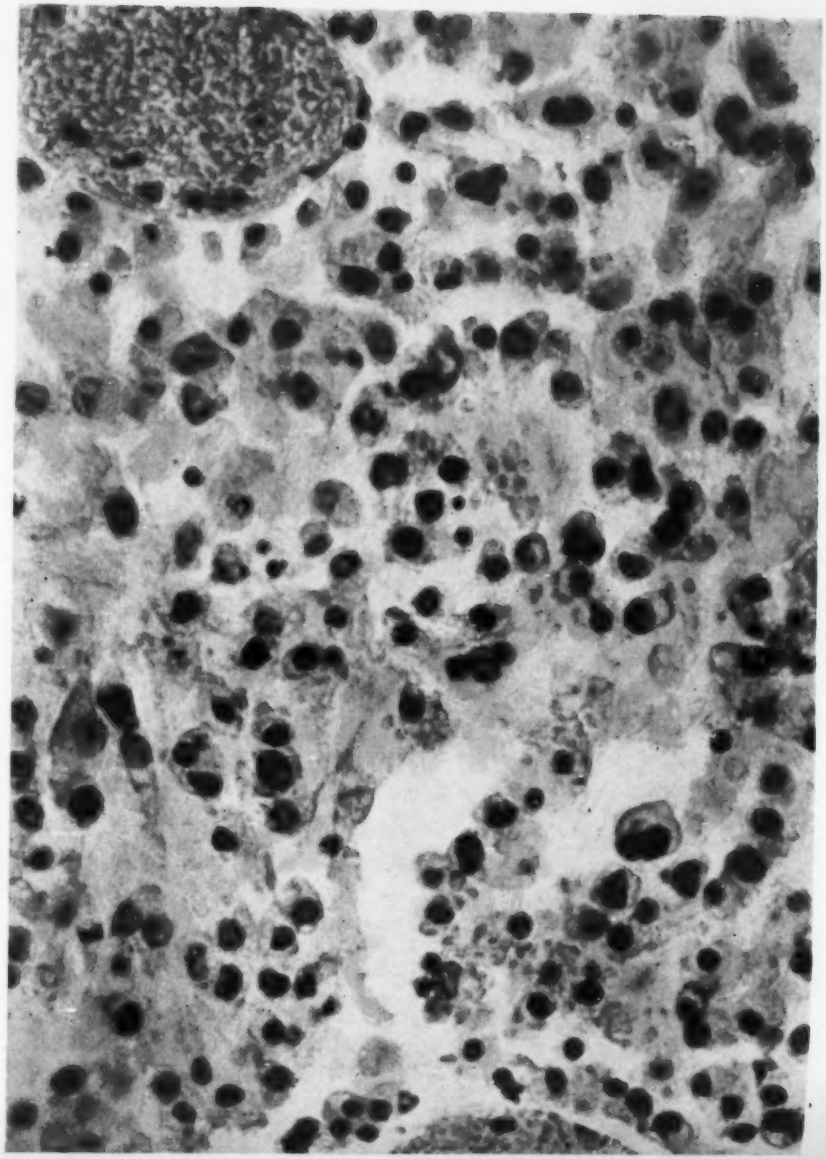

FIG. 3.—Sarcoma H.S.1, 14 days after implantation into the flank of a weanling rat treated with Cortrophin ZN, 5 units daily/rat intramuscularly for 14 days. Note the similarity of the tumour to that from the cortisone treated animal (*Fig.* 2). Magnification, × 350.

TABLE II

COMPARISON OF THE EFFECTS OF CORTISONE AND CORTROPHIN ZN ON THE GROWTH OF SARCOMA H.S.1 IN WEANLING RATS

See text for method of scoring tumour size.

Expt.	Treatment	No. of Rats	Size of Tumour 14 Days after Implantation								Total Score/Group
A	Cortisone acetate 20 mg./100 g. subcutaneously at time of implant plus 3 further injections at 48 hr. intervals	8	+ + + + +	+ + +	+ +	+ +	+ +	+ +	+	+	17
	Cortrophin ZN 5 units daily/rat intramuscularly for 14 days	8	+ + +	+ + +	+ +	+ +	+ +	+	+	+	15
B	Cortisone acetate 20 mg./100 g. subcutaneously at time of implant plus 3 further injections at 48 hr. intervals	8	+ + +	+ +	+ +	+ +	+ +	+ +	+	+	15
	Cortrophin ZN 5 units daily/rat intramuscularly for 14 days	8	+ + +	+ +	+ +	+ +	+	+	+	+	13

FIG. 3.—Sarcoma H.S.1, 14 days after implantation into the flank of a weanling rat treated with Cortrophin ZN, 5 units daily/rat intramuscularly for 14 days. Note the similarity of the tumour to that from the cortisone treated animal (Fig. 2). Magnification, 350.

FIG. 2.—Sarcoma H.S.1, 14 days after implantation into the flank of a weanling rat treated with cortisone acetate, 20 mg./100 g. body weight subcutaneously at the time of implant and 3 further doses at 48 hr. intervals. A multinucleated round cell sarcoma with extensive fibrous elements. Magnification, 350.

With the exception of deoxycortone acetate all the steroids promoted the growth of the sarcoma; from a comparison of the effective doses it was apparent that 9α fluorohydrocortisone was the most active steroid followed in order by prednisolone, then by hydrocortisone and cortisone which were equally active. However, apart from the smaller dosage necessary these steroids had no better effect than cortisone on the growth of the tumour. With large doses of the steroids, the rats lost body weight due to the catabolic effect of the hormones; some died probably because of a combination of this effect with the sudden flare-up of a dormant infection.

The Effect of Corticotrophin on Tumour Growth.—Experiments were also performed to determine the effect of corticotrophin. The first experiments with soluble corticotrophin failed to induce tumour growth, and a long-acting preparation, namely corticotrophin precipitated with zinc hydroxide (Cortrophin ZN, Organon), was used in order to ensure that the blood concentrations of the hormone were maintained. The preparation was administered daily by intramuscular injection for 14 days; the first dose was injected into the hind limb immediately after the tumour implantation. The results of two separate experiments are summarized in Table II, in which tumour growth in rats treated with Cortrophin ZN (5 units/rat daily) was compared with the growth of the tumour in rats receiving the standard cortisone treatment. Tumour sizes have been assessed by visual observation on a scale extending from 1 plus (+) to 4 plusses (++++), and a numerical score has been allotted to the results by summing the plusses. In early experiments, the tumours were weighed as well as being assessed visually, and it was found that there was good correlation between the two estimates of tumour size. It is apparent from these results that Cortrophin ZN, when injected daily, will promote tumour growth to the same degree as the cortisone treatment.

If the dosage of Cortrophin ZN was reduced to 2 units/rat/day tumour growth occurred, while with a dose of 1 unit daily the tumour sometimes commenced to develop but regressed while still quite small, usually about the ninth day after implantation. When the dose of the preparation was increased to 4 or 8 units/rat daily, tumour growth was only slightly better than that produced by the 2 unit dosage. These results are summarized in Table III; it is also evident that Cortrophin ZN caused a decrease in the body weight and an increase in the adrenal weights of the treated rats; both these effects were related to the dose of the preparation. It was also found that the administration of corticotrophin need not necessarily start immediately after implantation of the tumour; if the first dose of Cortrophin ZN

TABLE III

THE EFFECT OF VARIOUS DOSES OF CORTROPHIN ZN ON THE GROWTH OF SARCOMA H.S.1 IN WEANLING RATS

Cortrophin ZN was given intramuscularly daily for 14 days. The asterisk indicates that some of these tumours grew slightly for 7 days and then regressed.

Cortrophin ZN (Units)	No. of Rats	Tumour Growth	Mean Wt. of Rat (g. ± S.E.)	Mean Paired Adrenal Wt. (mg. ± S.E.)
8	8	Growth	40 ± 1·2	67·4 ± 6·8
4	8	,,	41 ± 1·7	58·2 ± 1·6
2	8	Slight growth	49 ± 2·0	31·2 ± 1·8
1	8	*No growth	78 ± 4·4	23·8 ± 1·0
Zinc suspension control	8	No growth	85 ± 3·8	12·4 ± 0·8

was delayed for a period of 1 day, or, on one occasion, 2 days after implantation, the tumour grew; if the delay between implantation and treatment was extended beyond 2 days then the tumour did not develop.

The histological appearance of the tumour removed from cortisone treated rats did not differ in any way from that removed from rats treated with Cortrophin ZN. In both cases (Figs. 2 and 3) microscopical examination of the tumour tissue revealed a multinucleated round cell sarcoma with extensive fibrous elements. This appearance was in accord with the findings of Toolan (1954).

Mode of Action of Corticotrophin on Tumour Growth.—A further series of experiments were carried out to investigate the mode of action of corticotrophin (Table IV). The tumour failed to develop in untreated adrenalectomized or hypophysectomized animals, but grew well if these animals were treated with cortisone. Corticotrophin was without effect in adrenalectomized animals, but promoted tumour growth after hypophysectomy. It appeared that the action of corticotrophin on tumour growth was by way of the adrenal cortex and was related to the enhanced adrenocortical secretion which it produced.

TABLE IV

THE EFFECT OF REMOVAL OF ENDOCRINE GLANDS AND REPLACEMENT THERAPY ON THE GROWTH OF SARCOMA H.S.1 IN WEANLING RATS

An asterisk indicates no evidence of tumour growth.

Procedure	Tumour Growth
Cortisone acetate 20 mg./100 g. body wt. ×4, subcutaneously ..	Growth
Cortrophin ZN 5 units daily/rat, intramuscularly for 14 days	,,
Adrenalectomy	No growth
,, +cortisone acetate	Growth
,, +Cortrophin ZN	No growth
Hypophysectomy	*Rats died
,, +cortisone acetate	Growth
,, +Cortrophin ZN	,,

In a number of experiments, the adrenal glands were removed from corticotrophin treated rats exhibiting tumours; doses of this hormone ranged from 1 unit to 20 units/rat daily for 14 days. As might be expected, the adrenal glands showed varying degrees of hypertrophy depending on the dose of corticotrophin administered. The glands were preserved in formol saline and subsequently weighed. In the same animals the size of the tumour was assessed and graded: no growth (−), partial growth (±) or positive growth (+ to ++++ and greater than ++++). An attempt to relate adrenal size with tumour growth was made. Fig. 4 shows that the tumour failed to grow in corticotrophin-treated rats when the combined weight of both adrenal glands was less than about 30 mg.; partial tumour growth was associated with glands slightly larger than this, while positive tumour growth occurred in animals with paired adrenals larger than about 50 mg. The paired adrenal glands from untreated rats weighed about 12 mg. These results indicate that there is a degree of correlation between adrenal enlargement and the growth of the tumour.

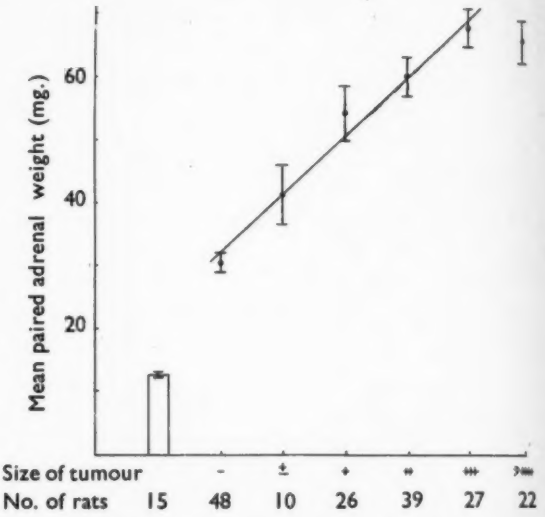

FIG. 4.—Relation between adrenal weight and tumour size in Cortrophin ZN treated rats. The regression line ($y=9.24\ x+22.97$) has an index of precision (λ)$=0.082$; the slope is very highly significant ($P<0.001$). Each point represents the mean paired adrenal weights of a group of animals, the vertical lines indicate the standard errors. Size of tumour is graded: no growth (−); partial growth (±); and positive growth (+ to ⩾ ++++). The histogram represents the mean paired adrenal weights of a group of untreated control rats.

DISCUSSION

The soft tissue sarcoma H.S.1 was taken from a patient about five years ago and has since been transplanted over 150 times in cortisone-treated hamsters, rats, and mice. The growth and development of the tumour were also promoted by treating the animals with adrenal steroids other than cortisone; 9α fluorohydrocortisone was effective in this respect at a dosage about one-tenth that of cortisone, while prednisolone was active at half the cortisone dosage. Hydrocortisone appeared to have similar activity to cortisone, but deoxycortone acetate did not induce tumour growth even when injected in very high dosage.

Soluble corticotrophin was not effective in promoting tumour growth; however, a long-acting preparation, Cortrophin ZN, was just as effective as cortisone at a dose of 2 units or more/rat/day.

The tumour failed to develop in untreated adrenalectomized or hypophysectomized rats but grew well if they were treated with cortisone; corticotrophin was without effect in adrenalectomized animals, but promoted tumour growth after hypophysectomy; thus it was apparent that the effect of Cortrophin ZN on tumour growth was mediated by the adrenal cortex and was related to the enhanced secretion of adrenocorticoids. The marked hypertrophy of the adrenal glands removed from Cortrophin ZN treated rats with tumours provided further evidence to support this view. In spite of the considerable variation in adrenal size, it was evident that up to a certain stage of adrenal enlargement there was a relation between tumour development and adrenal hypertrophy. Furthermore, rats which showed a positive tumour growth when treated either by cortisone or by corticotrophin all showed greatly diminished body growth. If the dose of Cortrophin ZN was too small to promote tumour growth, then body weight of the animal was not greatly diminished. This reduced body weight in the cortisone-treated animals is believed to be due to the catabolic action of the steroid (D'Arcy and Howard, 1958); in the case of the Cortrophin ZN treated rats it is likely to be due to a similar action of the enhanced adrenal output.

How exogenous or endogenous adrenocorticoids are able to break down the resistance of a host animal to the growth of malignant heterologous tissue is uncertain. Toolan (1955) suggested that cortisone may reduce the natural immunity of the host by reducing antibody function. The results obtained in this present work can add little to this discussion of the mechanism involved; however, two facts are clear. Firstly, age is a factor in the development of the resistance to the growth of heterologous tissue, and in older rats it is impossible to break down this resistance by the administration of adrenal steroids. Secondly, stimulation of the rat adrenal cortex by corticotrophin produces a sufficiently high blood titre of endogenous adrenocorticoids to enable the tumour to develop; this suggests that physiological adrenocortical stimulation may be a factor in the growth of the tumour.

There is a possible analogy between the regression of the H.S.1 tumour in rats when Cortrophin ZN treatment is stopped and the regression of some metastatic breast and prostate tumours after adrenalectomy. Although in the latter condition the patients normally receive cortisone replacement therapy, it is possible that the doses administered may be much less than the quantities of equivalent corticosteroid formerly secreted by the patients' own adrenal glands. It may be that the breast and prostate tumours which respond to adrenalectomy are those which are capable of producing an antibody or other defence mechanism on the part of the patient, and that this antibody formation is suppressed by the presence of the endogenous corticoids; such a mechanism by its emergence after adrenalectomy may be able to bring about a temporary cessation in the growth of the metastasizing tumour. On the other hand, in these cases it has been held that because the adrenal gland is an extraneous source of sex hormones, stimulating the growth of normal or malignant breast tissue, its removal may produce improvement because the output of such sex hormones is diminished. It is even possible that both of these mechanisms may have some bearing on the regression of the tumour.

The ability of corticotrophin to induce tumour growth, as shown in the present studies, may be correlated with a further clinical finding; namely in patients from whom the primary growth has been removed, there is a tendency for metastases to appear after subjection to stress, in particular to the surgical stress of a further operation. It seems likely that the stress-induced hypersecretion of corticotrophin, producing enhanced adrenocortical output, may be the contributory cause of this syndrome.

In view of the points of possible analogy between the clinical findings in the treatment of some cases of mammary cancer, and the present study of a human sarcoma growing in rats, it would seem to be profitable to investigate the extent to which any hyperactivity of a patient's own pituitary-adrenal system might influence the course of tumour development.

While this communication was awaiting publication, Palm, Teller, Mercker, and Woolley (1958) have shown that hydrocortisone had a similar effect to that of cortisone on the growth of the tumour H.S.1.

Our sincere thanks are due to Dr. Helene Wallace Toolan for her generous advice, and for supplying the H.S.1 tumour, and to Miss Jean Phillips for technical assistance. This work was financed by generous grants from the Wellcome Trust and the British Empire Cancer Research Fund.

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BRITISH PHARMACOLOGICAL SOCIETY

SUMMER MEETING, 16th to 18th JULY, 1959, AT THE DEPARTMENT OF PHARMACOLOGY, UNIVERSITY OF MANCHESTER

COMMUNICATIONS

1. **A. D. Forrest** and **K. Fotherby** (*introduced by* **C. R. B. Joyce**) (*Department of Psychological Medicine and M.R.C. Endocrine Research Unit, Edinburgh*).
The effect of chlorpromazine on adrenocortical function in humans.

2. **M. B. Gharpure** (*introduced by* **P. B. Marshall**) (*Department of Pharmacology and Therapeutics, Queen's College, Dundee*).
Effect of optical isomerism on the antihistamine and anti-acetylcholine actions of some phenothiazines. (Precirculated.)

3. **P. B. Marshall** (*Department of Pharmacology and Therapeutics, Queen's College, Dundee*).
Effect of sex hormones and gonadectomy on the sex difference in histamine excretion in the rat. (Precirculated.)

4. **L. G. Goodwin** (*The Wellcome Laboratories of Tropical Medicine, London, N.W.*1).
Pharmacologically active substances in animals infected with *Babesia rodhaini*.

5. **A. Nightingale** and **J. R. Trounce** (*Department of Experimental Medicine, Guy's Hospital Medical School, London, S.E.*1).
Some observations on intestinal muscle in Hirschsprung's disease.

6. **J. A. Hicklin** (*introduced by* **M. Weatherall**) (*Department of Pharmacology, London Hospital Medical College, E.*1).
Action of salicylates on potassium fluxes in rat diaphragms.

7. **M. I. Botros, E. Poulson** and **J. M. Robson** (*Department of Pharmacology, Guy's Hospital Medical School, London, S.E.*1).
The effects of 5-hydroxytryptamine and iproniazid on sexual development and pregnancy.

8. **W. D. M. Paton** (*Department of Pharmacology, Royal College of Surgeons of England, London*, W.C.1).
A kinetic hypothesis for the action of stimulant drugs and their antagonists.

9. **I. Khan, R. P. Stephenson** and **R. B. Barlow** (*Pharmacological Laboratory, University of Edinburgh*).
Amine oxidase and the actions of tryptamine and 5-hydroxytryptamine on the rat fundus and the rat uterus.

10. **P. F. D'Arcy, J. J. Grimshaw** and **R. W. Pickering** (*Allen and Hanburys Ltd., Ware, Hertfordshire*).
The action of some neuromuscular blocking drugs and other agents on ciliary movement.

11. **H. Blaschko** and **T. L. Chruściel** (*Department of Pharmacology, Oxford*).
Enzymatic decarboxylation of amino-acids related to Dopa and their awakening action in mice.

12. **G. E. Davies** and **J. S. Lowe** (*Imperial Chemical Industries Ltd., Pharmaceuticals Division, Macclesfield, Cheshire*).
Permeability factors released from guinea-pig serum by antigen-antibody precipitates.

13. **A. L. A. Boura** and **A. F. Green** (*The Wellcome Research Laboratories, Beckenham, Kent*).
The pharmacological properties of bretylium (Darenthin), a new hypotensive agent.

14. **A. L. A. Boura, A. F. Green** and **A. McCoubrey** (*The Wellcome Research Laboratories, Beckenham, Kent*).
On the mode of action of bretylium (Darenthin).

15. **Daphné Joyce** (*introduced by* **C. R. B. Joyce**) (*Academic Unit in Neurology, Institute of Neurology, Queen Square, London, W.C.*1).
Changes in brain 5-hydroxytryptamine content after death.

16. **C. R. B. Joyce** (*Department of Pharmacology, The London Hospital Medical College, London, E.*1).
Predicting the reactions of individuals to drugs and dummies.

17. **L. Golberg, L. E. Martin** and **P. Sheard** (*Benger Laboratories Ltd., Holmes Chapel, Cheshire*).
Some aspects of the pharmacology of chymotrypsin powder administered by inhalation.

18. **R. Hughes** and **L. Golberg** (*Benger Laboratories Ltd., Holmes Chapel, Cheshire*).
Aspects of analgesic testing in laboratory animals.

19. **L. C. Blaber** and **W. C. Bowman** (*introduced by* **G. A. H. Buttle**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).
The comparison between the effects of edrophonium (Tensilon) and choline in the skeletal muscles of the cat.

20. **B. B. Newbould** and **R. Kilpatrick** (*Department of Pharmacology and Therapeutics, University of Sheffield*).
The inhibitory effect of plasma proteins on the antibacterial activity of some new sulphonamides. (Precirculated.)

21. **T. D. Whittet** (*introduced by* **Sir C. Lovatt Evans**) (*University College Hospital, London, W.C.*1).
The unusual thermolability of the pyrogens of London tap water.

22. **J. H. Burn** and **M. J. Rand** (*Department of Pharmacology, Oxford*).
Effect of noradrenaline and related substances in restoring the action of tyramine in animals treated with reserpine.

23. **Elizabeth F. Marshall, G. S. Stirling, A. C. Tait** and **A. Todrick** (*introduced by* **J. J. Lewis**) (*Department of Clinical Research, Crichton Royal, Dumfries*).
The effect of iproniazid and imipramine on the blood 5-hydroxytryptamine in man.

24. **E. Letley** (*introduced by* **G. Brownlee**) (*Department of Pharmacology, University of London King's College, W.C.*2).
The modification by adrenaline of the acetylcholine-induced contracture of the denervated rat tibialis muscle.

25. **P. S. Guth** (*introduced by* **J. H. Gaddum**) (*Institute of Animal Physiology, Babraham, Cambridge*).
A possible mode of action for chlorpromazine.

26. **J. M. van Rossum, E. J. Ariens** and **P. C. Koopman** (*introduced by* **J. H. Burn**) (*Pharmacologisch Laboratorium, R. K. Universiteit, Nijmegen*).
Intrinsic activity and spare receptors. Theoretical consideration and experimental proof of spare receptors. (Precirculated.)

DEMONSTRATIONS

The following Demonstrations were given in the University of Manchester.

1. **A. Hughes** and **R. S. Tonks** (*Department of Pharmacology, Welsh National School of Medicine, Cardiff*).
How cortisone prevents hypersensitivity carditis. (Precirculated.)

2. **L. Pirbos** and **G. F. Somers** (*Distillers Company (Biochemicals) Ltd., Speke, Liverpool*, 24).
Gastric ulcers in mice and rats.

3. **L. Golberg, L. E. Martin** and **A. Batchelor** (*Benger Laboratories Ltd., Holmes Chapel, Cheshire*).
Tissue enzyme changes in animals following excessive parenteral dosage with iron.

4. **P. A. Nasmyth** and **P. N. Cardew** (*Pharmacology Department and the Photographic Department, St. Mary's Hospital Medical School, London, W.*2).
Noradrenaline and adrenaline (film).

5. **R. E. C. Altounyan** and **G. Starmer** (*Department of Pharmacology, The University, Manchester*).
Testing of analgesics in man.

6. **T. H. B. Bedford** (*Department of Pharmacology, The University, Manchester*).
The effect of movement of the head and neck in the vertical plane on the lumbar and cisternal cerebrospinal fluid pressures in the dog.

7. **J. H. Burn** and **M. J. Rand** (*Department of Pharmacology, Oxford*).
The sympathetic postganglionic mechanism.

8. **J. H. Burn** and **P. J. Goodford** (*Department of Pharmacology, Oxford*).
Cardiac fibrillation in relation to duration of the action potential.

9. **H. Jackson** (*Christie Hospital and Holt Radium Institute, Manchester*).
Actions and fate of alkylating agents.

10. **J. D. P. Graham** and **M. R. Gurd** (*Department of Pharmacology, The Welsh National School of Medicine, Cardiff*).
Adrenaline receptor in the cat uterus.

The following Demonstrations were given by members of Staff of Imperial Chemical Industries Ltd., Pharmaceuticals Division, at Alderley Park, Macclesfield, Cheshire.

11. **G. W. Ash** and **D. G. Davey**.
The establishment of a colony of pathogen-free animals.

12. **A. R. Martin** (*introduced by* **A. Spinks**).
Griseofulvin : the systemic and local treatment of experimental dermatophytosis.

13. **J. Dee** (*introduced by* **J. Raventos**).
A new varnish for kymograph tracings.

14. **J. Raventos.**
A simple method for recording the contractions of the uterus of small animals *in situ*.

15. **J. Raventos**.
A small bath for the isolated diaphragm preparation of the rat.

16. **G. E. Davies.**
The use of Coomassie Blue to indicate areas of increased capillary permeability and carageenin granulomata in rats.

17. **J. M. Thorp.**
Coomassie Blue, a new dye for determination of cardiac output and diagnosis of cardiac defects.

18. **G. B. Horsfall** (*introduced by* **A. Spinks**).
A transistorized cardiotachometer.

19. **G. B. Horsfall** (*introduced by* **A. Spinks**).
Cumulative impulse recorders.

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EFFECTS OF DIACETYL MONOXIME ON NEUROMUSCULAR TRANSMISSION

BY

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(RECEIVED DECEMBER 2, 1958)

The action of diacetyl monoxime on neuromuscular transmission has been studied in frogs, chickens, and cats, and in isolated rat phrenic nerve-diaphragm preparations. In frogs and chickens the oxime caused a flaccid paralysis; in chickens there was sometimes opisthotonos. In the indirectly stimulated rat diaphragm, diacetyl monoxime decreased the height of a single twitch, but a tetanus was well sustained. In cats, the twitch height of the indirectly excited gastrocnemius-soleus muscle was reduced by diacetyl monoxime more than was that of the tibialis anterior muscle, but in both muscles a tetanus was well maintained. Diacetyl monoxime reduced the response to direct stimulation of both the rat diaphragm and cat muscles. Diacetyl monoxime injected intra-arterially in the cat elicited a transient hypertension and a gasp. Diacetyl monoxime did not reverse the neuromuscular block caused by anticholinesterases either in isolated rat phrenic nerve-diaphragm preparations or in cats.

Amongst the oximes tested as antidotes against poisoning by organophosphorus insecticides, diacetyl monoxime has shown promising properties. Recently, Jager and Stagg (1958) have investigated the toxicity of diacetyl monoxime in man and Jager, Stagg, Green, and Jager (1958) its distribution in and excretion from the body. Wagley (1957) found that diacetyl monoxime decreased the end-plate potential in isolated amphibian muscle.

Since another active oxime, pyridine-2-aldoxime methiodide, is a neuromuscular blocking agent (Holmes and Robins, 1955; Fleisher, 1957), it was of interest to examine the effects of diacetyl monoxime on neuromuscular transmission.

METHODS

Frogs (*Rana ridibunda pallas*) of both sexes weighing 18 to 30 g. received injections into the dorsal lymph sac through the muscles of the hind limb, thus avoiding leakage of the injected solution. A volume of 0.04 ml./10 g. of body weight was used. Chickens weighing 90 to 140 g. of either sex, 3 weeks old, received injections (0.02 ml./10 g. of body weight) into the external jugular vein. All animals were observed for 24 hr. following injection.

Isolated phrenic nerve-diaphragm preparations (Bülbring, 1946), prepared from male white rats weighing 200 to 250 g., were placed in a 100 ml. bath containing Krebs solution at room temperature, bubbled with a mixture of 95% air and 5% CO_2.

Quantities of substances tested on this preparation are expressed in terms of final molar concentrations. Unless otherwise stated, indirect stimulation by rectangular pulses was of two kinds, either single supramaximal pulses of 0.5 msec. duration at a rate of 14/min. or tetanic stimuli at 350/min. applied for 5 sec. every 2 min. For direct muscle stimulation, single stimuli of 0.5 msec. duration were used.

Cats (of either sex) weighing 1.8 to 3.8 kg. were anaesthetized with ether followed by 80 mg./kg. of chloralose injected through a cannula in the external jugular vein. To record the contractions of the tibialis anterior and gastrocnemius-soleus muscles, the animals were prepared as described by Burn, Finney, and Goodwin (1950). The trachea was cannulated and spontaneous respiration was recorded from a rubber pneumograph tied around the chest and connected to a Marey tambour. The blood pressure was recorded from the right carotid artery with a mercury manometer; 20 mg. of heparin was injected to prevent clotting. About 15 to 20 min. before the injection of anticholinesterases, 0.5 mg. of atropine sulphate/kg. was administered intravenously and artificial ventilation was begun with a Palmer "Ideal" Pump. The vagosympathetic trunks were cut in the neck. Electrical stimulation of the distal end of the cut sciatic nerve was carried out as for the phrenic nerve-diaphragm preparation; for direct stimulation of the muscles, the rod transfixing the femur was connected to the cathode, and a small stainless steel hook, which gripped the muscle tendons, was connected to the anode.

The oxime and anticholinesterases were injected intra-arterially through a metal cannula placed in the left

femoral artery, the iliac arteries having been ligated. The concentrations of solutions were so adjusted to give single injections of a maximum volume of 2 ml. for the oxime and 1 ml. for the anticholinesterases. The injections were made within 10 sec. of each other. For continuous injection of the oxime, a Braun slow infusion apparatus delivered 600 μg./0.5 ml./min. over 30 min.

Solutions of pure diacetyl monoxime and pure ethyl pyrophosphate (TEPP) were made with 0.9% NaCl and used immediately. Neostigmine methylsulphate and tubocurarine hydrochloride were obtained ready for use in sterile saline and diluted when necessary. All doses are expressed in terms of body weights.

Results

Diacetyl monoxime (100 mg./kg.) caused a flaccid paralysis and loss of the righting reflex within 40 min. in one of four frogs. This state lasted 15 min., after which the animal recovered its normal mobility. With 200 mg./kg. all of four animals were paralysed for from 20 to 105 min. Similar results were obtained on repetition of the experiments.

Two chickens injected with diacetyl monoxime (50 mg./kg.) showed transient paresis and weakness almost immediately but recovered within 1 min. Ten chickens injected with 100 mg./kg. developed a flaccid paralysis but recovered within 1 to 6 min. In three of these, opisthotonos, lasting 1 to 2 min., was also seen.

Twenty experiments were carried out on phrenic nerve-diaphragm preparations. When the muscle was stimulated with single shocks, concentrations of 1.25×10^{-3} M to 3×10^{-2} M of diacetyl monoxime caused a graded decrease in the height of contraction. With 4×10^{-2} M, there was a complete block. Direct stimulation of the muscle during the block elicited a contraction (Fig. 1) and a tetanus was also well sustained. Diacetyl monoxime also enhanced the blocking effect of tubocurarine (Fig. 2).

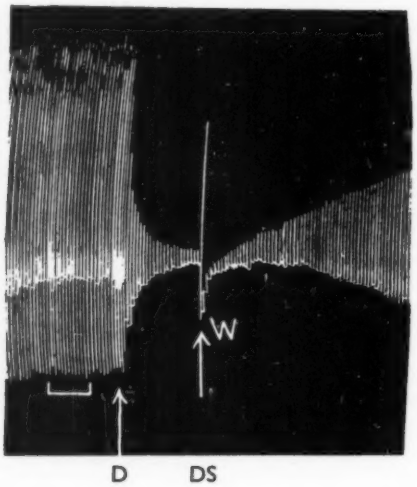

FIG. 1.—Effect of diacetyl monoxime on the isolated rat phrenic nerve-diaphragm preparation stimulated indirectly by single supramaximal stimuli, 0.5 msec., 14/min. At **D**, 1×10^{-2} M diacetyl monoxime was added; at **DS**, direct stimulation of the muscle. **W**, wash. Time, 1 min.

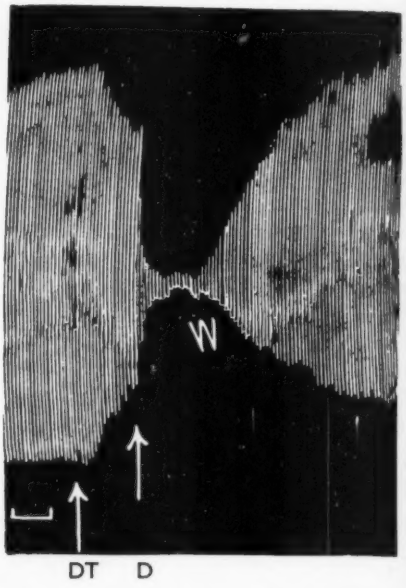

FIG. 2.—Isolated rat phrenic nerve-diaphragm preparation indirectly stimulated as in Fig. 1. Block caused by 1.27×10^{-6} M tubocurarine hydrochloride (**DT**) augmented by 1×10^{-2} M diacetyl monoxime (**D**). **W**, wash. Time, 1 min.

To examine the effect of diacetyl monoxime on the muscle fibre itself, experiments resembling those of Holmes and Robins (1955) were carried out. The preparations were maintained in a concentration of 1×10^{-2} M of diacetyl monoxime for 4 hr. and stimulated indirectly, with direct tetanic stimulation at intervals. With indirect stimulation there was an initial decrease in the height of contraction and no further change. The tetanus was always well sustained, and post-tetanic potentiation of the single twitch was seen. The response to direct stimulation was reduced after 2 to 3 hr. In another experiment in which the diaphragm, in 1×10^{-2} M diacetyl monoxime, was stimulated alternately directly and indirectly, the muscular response was also reduced, the indirect response being abolished before the direct.

In cats, single intra-arterial injections of 100 μg. and 5 mg./kg. of diacetyl monoxime had no effect

on the height of contraction of the tibialis anterior muscle, 5 mg./kg. produced only a slight effect on the response of the gastrocnemius-soleus muscles to single indirect stimuli. Doses of 30 mg./kg. of diacetyl monoxime invariably caused a decrease in the single twitch, the gastrocnemius-soleus muscle being affected more than the tibialis anterior muscle. Normal responses returned in 20 to 30 min. for the tibialis anterior muscle and 35 to 40 min. for the gastrocnemius-soleus muscle. With doses above 50 mg./kg., diacetyl monoxime completely blocked neuromuscular transmission in the gastrocnemius-soleus muscle and reduced activity in the tibialis anterior muscle to about 50% of normal. If, during the block, tetanic stimuli were applied the muscles gave a well-sustained response and a post-tetanic potentiation of the single twitch (Fig. 3). Direct stimulation of the muscles during the block caused a contraction.

After 30 mg./kg. of diacetyl monoxime a tetanus was always well sustained, although there was a marked reduction of the height of contraction of the gastrocnemius-soleus muscle and to a minor extent of the tibialis anterior muscle (Fig. 4). In some experiments, after the injection of 50 mg./kg. of diacetyl monoxime, the muscle responses to single and tetanic stimuli never completely recovered.

Effects on Blood Pressure and Respiration.—Single doses of 30 mg./kg. of diacetyl monoxime or more at times caused a transient rise in blood pressure of 30 to 70 mm. Hg (Fig. 3) followed by a brief hypotension (Fig. 4). After a deep gasp the respiration

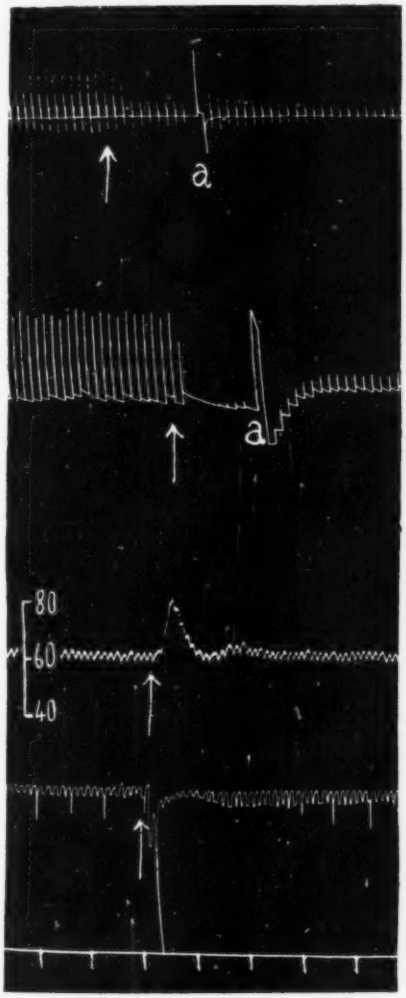

FIG. 3.—Cat, 2.5 kg., male. Records from above downwards: twitches of tibialis anterior muscle; twitches of gastrocnemius-soleus muscle; blood pressure (mm. Hg); respiration (inspiration downwards); time, 30 sec. Indirect stimulation (0.5 msec., 14/min.). At arrows, intra-arterial injection of diacetyl monoxime, 50 mg./kg.; at a, well-sustained contracture with tetanic stimulation for 5 sec. at 350/min.

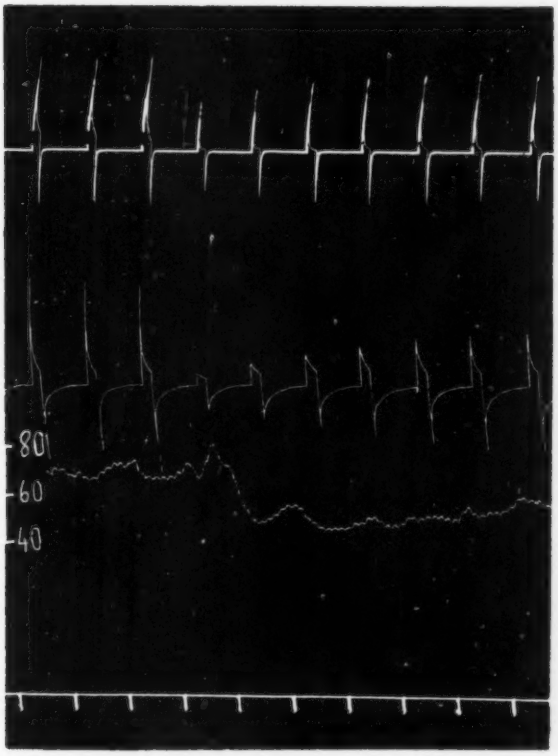

FIG. 4.—Cat, 1.8 kg., female. Records from above downwards: twitches of tibialis anterior muscle; of gastrocnemius-soleus muscle; blood pressure (mm. Hg); time, 30 sec. Indirect tetanic stimulation for 5 sec. every 30 sec. at 350/min. At arrow, intra-arterial injection of diacetyl monoxime, 30 mg./kg.

was usually rapid and shallow (Fig. 3) but sometimes continued normally. These responses, observed in 15 animals, have not been investigated further.

Effect of Diacetyl Monoxime on the Neuromuscular Block Caused by Anticholinesterases.—The action of diacetyl monoxime on the neuromuscular block caused by anticholinesterases was examined on isolated rat-diaphragm preparations and in cats.

In the rat diaphragm, two separate doses of ethyl pyrophosphate giving a total concentration of 1.7×10^{-5} M caused the usual enhancement followed by a decrease in the single twitches. In this latter state, diacetyl monoxime resulted in a further decrease (Fig. 5). When, instead of ethyl pyrophosphate, two doses totalling 6×10^{-6} M neostigmine methylsulphate were given, addition of diacetyl monoxime had the same result. During the period of enhancement of contraction, diacetyl monoxime antagonized ethyl pyrophosphate (Fig. 6). When two doses totalling 1.4×10^{-6} M ethyl pyrophosphate were added after 1×10^{-2} M diacetyl monoxime, the block caused by diacetyl monoxime was not reversed.

In another series of experiments, the diaphragm was stimulated tetanically every 2 min. When three doses giving a total concentration of 6×10^{-7} M ethyl pyrophosphate had been added at the start, after 7 and after 9 min., the muscle became unable to sustain the tetanus. Diacetyl monoxime (1×10^{-2} M) was then added and after 3.5 min. the preparation was washed. No important difference was found in the times required for the recovery of the tetanic response and that required without diacetyl monoxime, the times being 1 hr. 45 min. and 1 hr. 55 min.

With cats two types of experiment were carried out. In the first, 0.1 mg./kg. of ethyl pyrophosphate was injected, the sciatic nerve being given single stimuli. When the height of contraction of the tibialis anterior and gastrocnemius-soleus muscles had decreased, a single dose either of 100 μg. or of 5 mg./kg. of diacetyl monoxime was administered. In the second, tetanic stimulation was applied every 30 sec. and 0.25 mg./kg. of ethyl pyrophosphate was injected. When the muscles had lost their ability to sustain the tetanus, diacetyl monoxime was given as above or a slow infusion at 600 μg./0.5 ml./min. was given for 30 min. Control experiments in which ethyl pyrophosphate (0.25 mg./kg.) alone was injected showed that the tetanus was fully maintained again after 40 to 55 min. Sometimes, however, the recovery was not complete. In none of the above experiments did diacetyl monoxime influence the spontaneous recovery to normal height of single twitches or the ability to sustain a tetanus (Fig. 7).

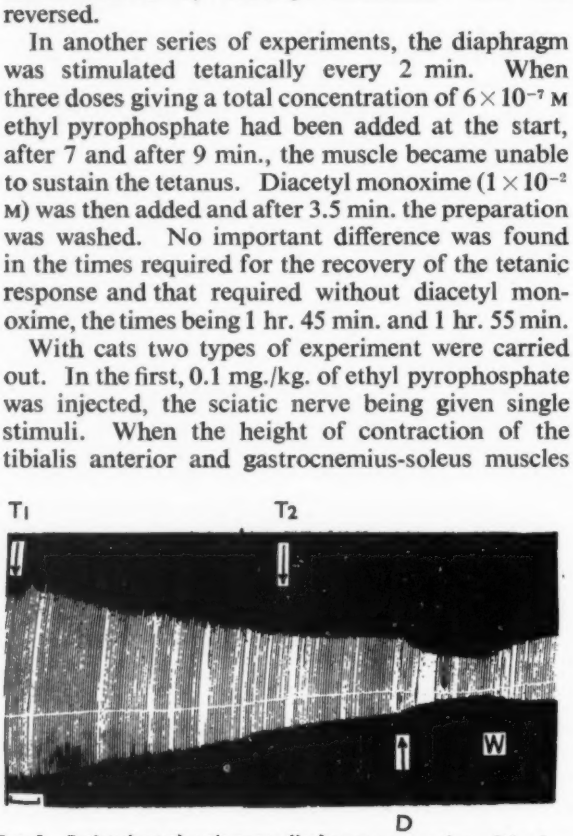

FIG. 5.—Isolated rat phrenic nerve-diaphragm preparation. Stimulation as in Fig. 1. At T_1 and T_2: 8.6×10^{-6} M and 1.7×10^{-5} M ethyl pyrophosphate respectively; at **D**, 1×10^{-2} M diacetyl monoxime. W, wash. Time, 1 min.

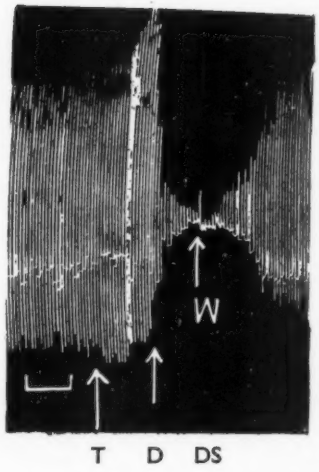

FIG. 6.—Rat phrenic nerve-diaphragm preparation. Stimulation as in Fig. 1. At **T**, 1.4×10^{-4} M ethyl pyrophosphate showing enhancement of single twitch. At **D**, 1×10^{-2} M diacetyl monoxime showing antagonism. At **DS**, contraction of the muscle after direct stimulation. **W**, wash.

DISCUSSION

The experiments showed that diacetyl monoxime, like pyridine-2-aldoxime methiodide, acted as a neuromuscular blocking agent in frogs, chickens, and cats and on the isolated rat phrenic nerve-diaphragm preparation. Grob and Johns (1958) have stated that diacetyl monoxime has no such effect in man. The type of block appeared to be of the competitive rather than the depolarizing type, but had some properties of the latter: for example, a flaccid paralysis was observed in all the chickens, but in some there was also opisthotonos. Two

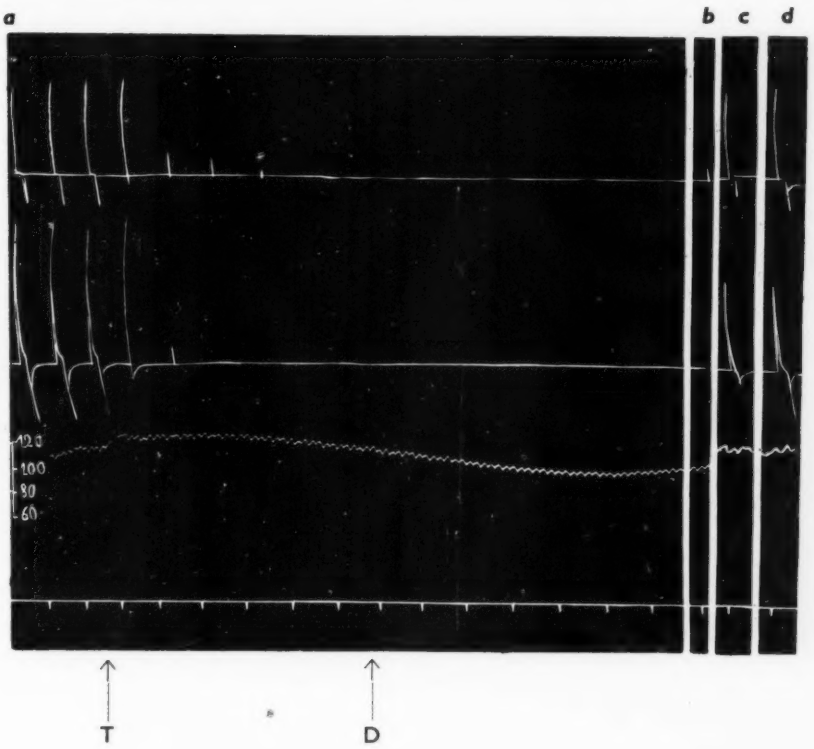

FIG. 7.—Cat, 2.0 kg., female, given atropine and artificially ventilated. Records from above downwards: tibialis anterior muscle; gastrocnemius-soleus muscle; blood pressure (mm. Hg); time, 30 sec. Indirect tetanic stimulation for 5 sec. every 30 sec. at 350/min. At **T**, ethyl pyrophosphate (0.25 mg./kg.) intra-arterially causing a gradual loss of ability to maintain a tetanus. At **D**, start of continuous intra-arterial infusion of diacetyl monoxime (600 μg./0.5 ml./min. for 30 min.). At ***b***, ***c***, and ***d***, tracings obtained 10, 34, and 53 min. after commencement of diacetyl monoxime infusion. Note that diacetyl monoxime did not influence the spontaneous recovery of the ability to maintain tetanus.

effects of diacetyl monoxime upon the striated muscle of the same human subject have been reported by Jager and Stagg (1958), who found clonic movements of the head at the same time as flaccid paralysis of the limbs.

In cats, the gastrocnemius-soleus muscle was more affected than the tibialis anterior muscle, as with curare-like drugs, but a tetanus was always well sustained. In the isolated diaphragm preparation, diacetyl monoxime decreased the twitch both during the enhancement and subsequent block caused by anticholinesterases.

Diacetyl monoxime appears to act mainly at the end-plate region, as direct muscle stimulation always elicited a contraction both in the isolated preparation and the anaesthetized animal. Some effect on the muscle fibre itself is also indicated, however, because sometimes the initial height of contraction was not fully regained.

It is well known that anticholinesterases first cause an enhancement and later a decrease or block of twitch responses in striated muscle stimulated indirectly. The ability to sustain a tetanus is also suppressed. Monoisonitrosoacetone (Holmes and Robins, 1955) and pyridine-2-aldoxime methiodide (Holmes and Robins, 1955; Wills, Kunkel, Brown, and Groblewski, 1957), relieve the neuromuscular block caused by anticholinesterases. Grob and Johns (1958) have reported that diacetyl monoxime in minute quantities (50 to 100 μg.) promptly and strikingly reverses the local neuromuscular block produced by anticholinesterases in man. With these and even larger quantities, this effect of diacetyl monoxime in cats and rat phrenic nerve-diaphragm preparations was not observed here. Holmes and Robins (1955) have stated that the reversal of neuromuscular block is entirely due to a reactivation of inhibited cholinesterase in the muscle. Since diacetyl monoxime is a poor reactivator compared with pyridine-2-aldoxime methiodide and monoisonitrosoacetone, its failure to reverse the block, which is a peripheral phenomenon, is not surprising. Thus diacetyl monoxime appears to have a more marked central than peripheral action in protecting against anticholinesterase poisoning. The same authors have also found that some oximes given close-intra-arterially failed to reverse the neuromuscular block elicited by anticholinesterases, because the oximes were not " fixed " in the muscle. This does not seem to be true of diacetyl monoxime, since a relatively small single dose (5 mg./kg.) caused a slight decrease in the twitches, indicating that the substance was " fixed."

The ability of diacetyl monoxime to penetrate the blood-brain barrier has been demonstrated by Jager *et al.* (1958); this possibly explains why it acts as a synergist to pyridine-2-aldoxime methiodide (Edery and Schatzberg-Porath, 1958). With regard to tubocurarine, diacetyl monoxime appears to act differently from the oximes used by Holmes and Robins (1955), for they found that these oximes have no effect on the block caused by tubocurarine. In our experiments diacetyl monoxime and tubocurarine were synergic.

In cats the rise in blood pressure produced by a single injection of diacetyl monoxime, which was found by Jager and Stagg (1958) in man, was also observed here.

My thanks are due to Mrs. G. Schatzberg-Porath for her help.

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Brit. J. Pharmacol. (1959), **14**, 323.

THE ACTION OF TUBOCURARINE ON CILIARY MOVEMENT

BY

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(RECEIVED JANUARY 21, 1959)

Further investigation of the effect of tubocurarine on the transport of particles by cilia of the frog oesophagus has confirmed that, when Ringer solution buffered with bicarbonate was used, tubocurarine inhibited the movement in a concentration of 10^{-6} g./ml. If, however, Ringer solution with a phosphate buffer was used tubocurarine was without effect. The difference in action is probably due to changes in calcium ion concentration caused by the phosphate in the Ringer solution.

Conflicting evidence has arisen from studies on the effect of tubocurarine on ciliary movement in the frog oesophagus. Kordik, Bülbring, and Burn (1952) found that tubocurarine in a concentration of 10^{-6} g./ml. inhibited ciliary movement. However, Hill (1957) described experiments in which tubocurarine had no effect on ciliary movement even in concentrations as great as 2×10^{-3} g./ml. Hill (1957) suggested that the results of Kordik *et al.* were artefacts arising from their experimental methods. As a result of the observations of Hill (1957), Burn and Day (1958) made a further study of the action of tubocurarine. They found that tubocurarine in a concentration of 10^{-4} g./ml. decreased the rate of ciliary movement in seven consecutive experiments, and in six of these the rate of ciliary movement returned to its initial value when the tubocurarine was removed. They also showed that it was possible to identify unknown solutions of tubocurarine and to distinguish them from Ringer solution by the action on ciliary movement.

Further experiments have now been made on the effect of tubocurarine in the concentration used by Kordik *et al.* (1952), namely 10^{-6} g./ml. Experiments have also been carried out to try to account for the conflicting results previously mentioned.

METHODS

The oesophagus of the frog was prepared in a way similar to that described by Burn and Day (1958), except for a few modifications in the dissection. The frog was decapitated, removing the upper jaw immediately behind the eye sockets. The lower jaw was left intact. The spinal cord was destroyed and the frog was placed prone on a small cork mat. The skin was slit along the mid-dorsal line and the vertebral column and dorsal body wall removed as far back as the sacral hump. The forelegs were removed with all but the central section of the pectoral girdle. The oesophagus was then cut down the mid-dorsal line and the cut continued a short distance into the stomach wall. The oesophagus was pinned out as a roughly rectangular sheet, being kept taut by pins. It was kept as level as possible by placing pieces of cotton wool soaked in Ringer solution beneath it. In female frogs the ovaries and oviducts were usually removed as these were liable to get in the way. In some experiments the whole of the hindquarters and the viscera were removed.

The rate of ciliary movement was measured by observing movement of particles. The preparation was placed inside a Perspex box similar to that previously described (Burn and Day, 1958). Particles were dropped on to the membrane through a slit in the lid and the time taken to travel 0.5 or 1.0 cm. was measured. This was facilitated by observing the particles as they travelled between three parallel lines on the lid, ruled 0.5 cm. apart. To avoid errors of parallax a sight was fitted above the lines. The particles were poppy seeds which could pass through a sieve of 40 mesh but were retained by one of 60 mesh and were therefore between 0.423 and 0.635 mm. in diameter. In the experiments with a totally immersed membrane, particles of garnet which could pass through a sieve of 60 mesh (0.423 mm.) were used as the poppy seeds often floated to the top. The Perspex box and all the solution were placed throughout the whole of each experiment in a water bath kept thermostatically at 20°.

To avoid errors, a strict sequence of events was adhered to. The membrane was rinsed with Ringer solution and, exactly 5 min. later, ten particles were individually timed over the measured distance. This took about 7 min. The membrane was then rinsed again with Ringer solution and exactly 5 min. later

ten more particles were timed. This sequence of events was continued until there was a steady rate of transport; the membrane was rinsed with Ringer solution containing the drug under test and exactly 5 min. later the particle transport time was measured. A further application of Ringer solution plus drug could then be made or the membrane rinsed with drug-free Ringer solution and particle transport time measured again. This sequence, alternating the administration of plain Ringer and Ringer solution containing the drug, could be continued for as long as was required. The average rate of particle transport for each set of 10 particles was expressed in cm./min.

The preparations usually remained active for about 24 hr., but measurements were made only on newly set up preparations. Measurements on particles falling near the cut edges or in the centre of the preparation near the glottis were not included in the results.

Experiments were also carried out using an isolated membrane immersed in Ringer solution and this preparation behaved in exactly the same way as the other.

The five solutions were as follows, the concentrations being mM.: (1) bicarbonate Ringer solution (Kordik *et al.*, 1952): NaCl 119, KCl 1.9, $CaCl_2$ 1.1, $NaHCO_3$ 2.4; (2) phosphate Ringer solution (Hill, 1957): NaCl 115, KCl 2.0, $CaCl_2$ 1.8, buffered with Na_2HPO_4 2.0 mM. to *p*H 7.0; (3) NaCl 115, KCl 2.0, $CaCl_2$ 1.8; (4) NaCl 115, KCl 2.0, $CaCl_2$ 1.8, $NaHCO_3$ 10.0; (5) NaCl 115, KCl 2.0, $CaCl_2$ 1.8, $NaHCO_3$ 10.0 buffered with 4.1% CO_2. Solutions (3) to (5) were also used by Hill (personal communication).

Results

The effect of tubocurarine (10^{-6} g./ml.) was observed in eight experiments using bicarbonate Ringer solution (Kordik *et al.*, 1952). In all, a decrease in particle movement was observed after the addition of tubocurarine. After the tubocurarine had been removed the rate returned to that observed initially (Fig. 1 and Table I).

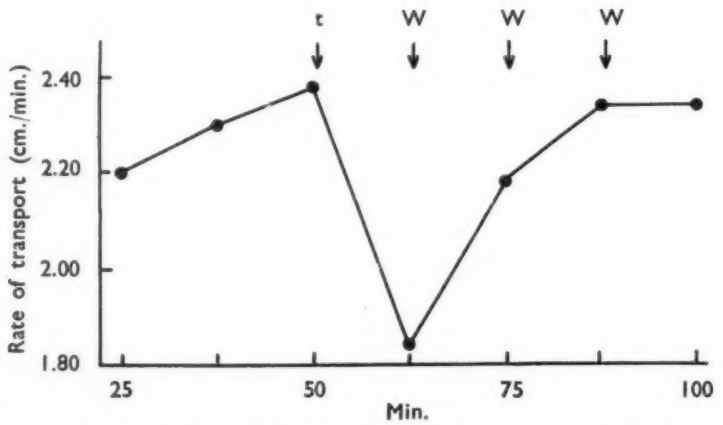

FIG. 1.—Action of tubocurarine (10^{-6} g./ml.) on ciliary movement in bicarbonate Ringer solution. Drug added at t. Washed with Ringer solution at W.

TABLE I

RATE OF PARTICLE TRANSPORT BY CILIA OF FROG OESOPHAGUS IN BICARBONATE RINGER SOLUTION CONTAINING TUBOCURARINE (10^{-6} G./ML.)

The rates are given as cm./min.

Initial	In Presence of Drug	After Removal of Drug
1·07	0·62	0·91
1·16	0·92	1·17
1·17	1·05	1·34
1·34	1·14	—
1·39	1·03	1·78
1·58	1·27	1·53
1·98	1·46	1·85
2·29	1·86	2·29
Mean 1·50	1·17	1·55

If the initial rate in each experiment is taken as 100, the relative rates before, during the presence of tubocurarine and after its removal may be directly compared. The mean rate during the action of tubocurarine was 78% and after removing the tubocurarine it was 103%.

Hill (1957) was unable to demonstrate the stimulating action of low concentrations of acetylcholine and two experiments were therefore carried out using 10^{-6} g./ml. of acetylcholine. The stimulation was shown quite clearly (Table II).

TABLE II

EFFECT OF ACETYLCHOLINE 10^{-6} G./ML. ON RATE OF PARTICLE TRANSPORT

Rates are given as cm./min. in the two experiments.

Initial	Rate in Presence of Drug	After Removal of Drug	In Presence of Drug
0·24	0·45	0·27	0·40
1·24	1·52	1·01	

The results showed the inhibitory effect of tubocurarine and the stimulating effect of acetylcholine on ciliary movement. The failure of Hill (1957) to observe these effects was therefore puzzling and so an attempt was made to explain her results. She had suggested that artefacts had arisen in the experiments of Kordik *et al.* (1952) due to the presence of counter currents and the accumulation of mucus. In her experiments she used a membrane immersed in Ringer solution which, it was claimed, prevented artefacts. However, experiments with her method still showed the inhibitory action of tubocurarine.

A further study of the report of Hill (1957) showed one difference which had previously been overlooked. This was that, whereas Kordik *et al.* (1952) had used a solution containing bicarbonate, Hill (1957) usually used a solution containing phosphate. As this appeared to be the only real difference between the experimental procedures, observations were made using the solution described in her paper. In neither of the first two experiments had tubocurarine an appreciable effect (Table III).

TABLE III

RATE OF PARTICLE TRANSPORT SHOWING ABSENCE OF EFFECT OF TUBOCURARINE (10^{-6} G./ML.) IN PHOSPHATE RINGER SOLUTION

Rates are given in cm./min.

Initial Rate	In Presence of Drug	After Removal of Drug
1·59	1·57	1·34
2·35	2·39	2·24

Next, both the Ringer solutions were used on one preparation. In the first experiment, the bicarbonate Ringer solution was used initially and tubocurarine exerted an inhibitory effect; when the Ringer solution was changed for the one containing phosphate it was found after equilibration that tubocurarine was no longer inhibitory. In the second experiment, the phosphate Ringer solution was used initially and tubocurarine had no effect; on changing to bicarbonate Ringer solution the inhibitory effect of tubocurarine was readily observed (Fig. 2).

These results explained the inability of Hill (1957) to observe the effect of tubocurarine using the phosphate Ringer solution described in her paper. They did not, however, explain why this failure occurred. Further experiments were therefore carried out on the effect of *p*H.

The *p*H of the Ringer solution used by Hill (1957) was 7.0 whereas that of Kordik *et al.* (1952) varied between 7.6 and 8.3 owing to the very low buffering power of the bicarbonate. It should be remembered, however, that the *p*H of frog blood is about 8.0. Tubocurarine in concentrations of 10^{-4} g./ml. or less did not alter the *p*H of either solution.

In bicarbonate Ringer solution which had been brought to *p*H 7.0 with HCl, the cilia showed normal activity and were still active 20 hr. after being set up. Tubocurarine 10^{-6} g./ml. caused marked inhibition (33%). When the *p*H of the phosphate Ringer solution was raised to 8.0, visible precipitation of calcium phosphate gradually occurred. The cilia were so depressed and irregular in their rate of beat when using this solution that no measurements were possible.

These observations give no indication that differences of *p*H were responsible for the different results. However, the precipitation of calcium suggested that reduced calcium ion concentration due to binding with the phosphate might be responsible. In this connexion it was found that calcium-free bicarbonate Ringer solution caused an increase in rate at the start followed by slowing and irregularity in rate. Tubocurarine had no significant effect under these conditions.

The three other Ringer solutions used by Hill (1957) were also tested. With each, tubocurarine

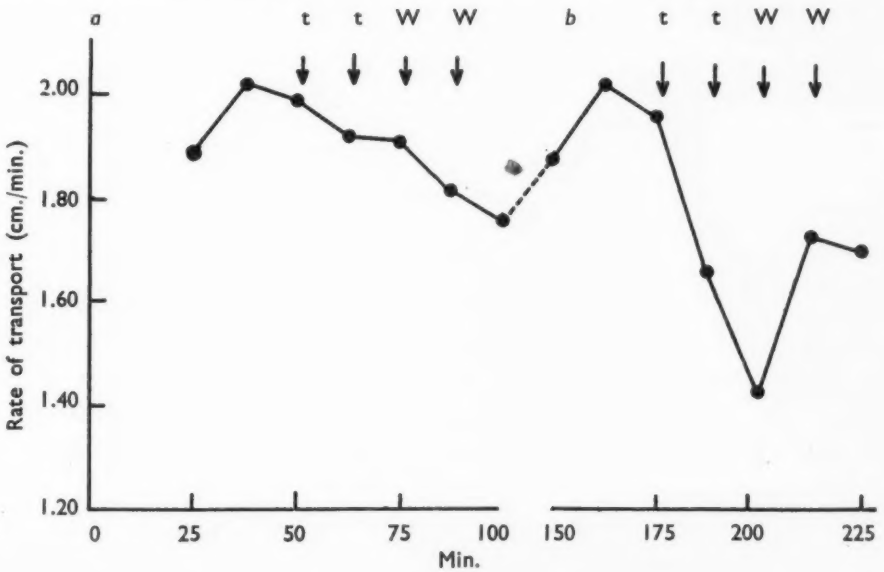

FIG. 2.—Action of tubocurarine (10^{-6} g./ml.) on ciliary movement on the same preparation, *a*, in phosphate and, *b*, bicarbonate Ringer solutions. Drug added at t. Washed with Ringer at W. Solution changed between 100 and 150 min.

caused a decrease in rate of particle transport (Table IV) and in two experiments the transport of particles was arrested altogether.

TABLE IV

RATE OF PARTICLE TRANSPORT IN PHOSPHATE-FREE RINGER SOLUTION (HILL, 1957)

Concentration of tubocurarine 10^{-5} g./ml. The rates are given in cm./min.

Sol. No.	Initial Rate	After Addition of Drug		Rate After
		3 min.	10 min.	
3	1·78	1·21	1·36	1·89
3	2·08	2·10	0	0
4	1·96	1·09	0	0
5	1·86	1·60	1·28	2·17

DISCUSSION

The results in this paper confirm the finding of Kordik *et al.* (1952) that tubocurarine (10^{-6} g./ml.) caused inhibition of the ciliary movement of the frog oesophagus. In the practical class of this Department, students observe the acceleration of ciliary movement by low concentrations of eserine and the inhibition by low concentrations of atropine. Frog Ringer solution containing bicarbonate as buffer is usually used, but on one occasion a solution containing phosphate as buffer, which had already been prepared for another purpose, was used instead. Eserine then showed no accelerating effect, though atropine was effective. This led to an investigation of the solution used by Hill (1957) which contained phosphate, and it was found that when phosphate was present the inhibitory action of tubocurarine was no longer seen; however, the action was restored in the same preparation when bicarbonate replaced the phosphate.

Dr. H. Blaschko pointed out that Alt (1930) had found that the phosphate interfered with tissue respiration. Thus even the inhibition of the respiration of kidney and liver slices by cyanide was reduced from 98% to 11% when bicarbonate buffer was replaced by a phosphate buffer. Alt (1930) suggested that this result might be due to the binding of calcium ions by the phosphate. This explanation might apply to the cilia, since, when calcium-free (bicarbonate) Ringer solution was used, a rapid depression followed an initial rise in rate and tubocurarine had no appreciable effect.

The use of Ringer solution containing phosphate in the majority of the experiments of Hill (1957) would therefore explain her failure to confirm the inhibitory action of tubocurarine. However, she also failed to observe the inhibitory action when using a bicarbonate Ringer solution. This failure together with the failure to observe the stimulant action of weak solutions of acetylcholine stands in contrast to the observations of Kordik *et al.* (1952), Bülbring, Burn, and Shelley (1953), Burn and Day (1958), and those reported here in which both these effects have been observed.

Attention has been drawn to the point that the rate of particle transport may be affected by mucus secretion. Hill (1957) stated that, during an experiment, the viscosity of mucus increases until it becomes so tenacious that the cilia can no longer propel particles. She suggested that the rapid cessation of flow observed by Dalhamn (1956) was because he did not moisten the surface. This could not account for the slowing with tubocurarine, for the irrigation of the mucous membrane whether with Ringer solution containing tubocurarine or with Ringer solution alone was performed at regular intervals of about 12 min., and moreover after removal of tubocurarine the original rate was restored.

Kordik *et al.* (1952) investigated the action of tubocurarine on salivary secretion in the cat and found that it had less than 1% of the paralysing action of atropine. Nevertheless tubocurarine was rather more potent than atropine in inhibiting transport by the cilia of the frog oesophagus. Bülbring *et al.* (1953) also reported that tubocurarine slowed the rate of the ciliary beat (observed by a stroboscope) on the edge of an isolated gill filament of *Mytilus edulis* washed by a flow of sea water. In these circumstances mucus secretion could not occur.

I wish to thank Professor J. H. Burn for his guidance throughout this work. I am indebted to Dr. H. Blaschko for his many helpful ideas.

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THE FATE OF OXYTOCIN IN MALE AND FEMALE RATS

BY

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A method for quantitative extraction of oxytocin from blood is described. The disappearance of injected oxytocin from the circulation in rats was shown to depend upon its uptake in the kidneys and in organs of the splanchnic vascular area. In lactating rats there was uptake by an additional organ or tissue, probably the mammary gland. In animals without kidneys or lactating mammary glands and with no circulation in the splanchnic area oxytocin was distributed into a volume greater than the extracellular fluid volume, and after equilibration the concentration in plasma did not change significantly. During severe haemorrhage increased amounts of antidiuretic activity were detected in blood when there was no significant increase in oxytocic activity.

Many peptide hormones, for example, insulin, corticotrophin, and vasopressin, disappear rapidly from the circulation (Elgee and Williams, 1954; Richards and Sayers, 1951; Greenspan, Li, and Evans, 1950; Ginsburg and Heller, 1953a). In the case of vasopressin, this has been shown clearly to be due to the rapid uptake of the hormone from the blood by the kidneys and the liver, rather than to irreversible inactivation of the hormone in the circulating blood (Ginsburg and Heller, 1953a; Crawford and Pinkham, 1954; Dicker, 1954; Ginsburg, 1957).

Jones and Schlapp (1936) and more recently Chaudhury and Walker (1957) have shown that oxytocin, also, has a short lifetime in the circulation. These observations have been confirmed in the work described in the present paper and extended by an analysis of the parts played by specific organs and tissues in the removal of oxytocin from the circulation. A preliminary report of this work has been made (Ginsburg and Smith, 1958).

METHODS

Adult albino rats of both sexes (180 to 250 g.) were used.

Assay of Antidiuretic Activity.—The method of Ginsburg and Heller (1953b) was used except that the bladders of the rats were cannulated and measurements of urine volume were taken at 5 min. intervals. Where the amount of antidiuretic activity in the test samples permitted, (2 and 2) dose assay was used.

Assay of Oxytocic Activity.—Uteri from rats which were found by vaginal smear examination to be in pro-oestrus or early oestrus were suspended and superfused according to the method of Gaddum (1953). The constant temperature of the superfusing fluid was within the range 30 to 33°, depending on the reactivity of the uterus; the rate of flow was 3 to 4 ml./min. and the composition of the fluid was NaCl 4.0 g./l., KCl 0.42 g./l., $CaCl_2$ 0.06 g./l., $MgCl_2$ 0.005 g./l., glucose 0.25 g./l., $NaHCO_3$ 0.50 g./l., sucrose 58.6 g./l., and atropine sulphate 1 mg./l., saturated with 95% O_2 and 5% CO_2.

Extraction of Oxytocin from Whole Blood.—Blood was collected into chilled polyethylene tubes from a cannulated carotid artery in rats under ether anaesthesia. The rats had been given previously 100 U./100 g. body weight of heparin by intravenous injection. The blood was centrifuged at 3,500 rev./min. for 30 min. at 5°. The plasma was separated, placed in chilled glass centrifuge tubes and 10 vol. of dry acetone were added immediately. After centrifugation for 10 min. at 2,500 rev./min., the supernatant fluid was separated and the precipitate washed once with an acetone-water mixture (9 parts acetone to 1 part water). The acetone in the combined supernatant fluid and washings was evaporated in a stream of air at 40°. The cloudy aqueous residue was extracted twice with 7 vol. of diethyl ether to remove traces of acetone and other ether-soluble material. Ether, which had dissolved in the aqueous phase, was evaporated in a stream of air at 40° leaving a clear solution. In one series of experiments, the sodium and potassium concentrations in such extracts were estimated using a Lange flame photometer. The results of these experiments were used to determine the amounts of NaCl and KCl to be added to an extract to make its electrolyte composition similar to that of the superfusion fluid.

Inulin was estimated by the method of Schreiner (1950).

The preparations used were oxytocin (Pitocin, Parke, Davis), vasopressin (Pitressin, Parke, Davis), heparin (Pularin, Evans) and inulin (British Drug Houses).

Results

Reactivity of the Rat Uterus in Superfusion Fluid containing Different Concentrations of NaCl.—In preliminary experiments in which the uteri were superfused with Ringer-Locke solution (for rat uteri) described in the 1953 British Pharmacopoeia it was often necessary to apply as much as 1 mU. oxytocin/ml. to elicit a contraction of the uterus and also tachyphylaxis occurred. As Hughes, McDowall, and Soliman (1956) have shown that this tachyphylaxis may be prevented or retarded by reducing the concentration of sodium in the fluid bathing an isolated smooth muscle, the effect of using a superfusion fluid with reduced sodium concentration was investigated.

Fig. 1 shows the effect of changing the concentration of sodium chloride from 0.9 g./100 ml. to 0.4 g./100 ml. upon the action of oxytocin on a uterus. The concentration of the other ingredients of the superfusion fluid was unaltered, but 58.5 g. of sucrose/l. was added to the solution containing less NaCl, to maintain isotonicity. During each application of oxytocin the flow of superfusion fluid was stopped, 0.2 ml. of the test solution was applied in 10 sec. and allowed to remain in contact with the uterus for a further 10 sec. before the flow was restarted. When the B.P. solution was used, the contractions produced by application of 0.1 mU. of oxytocin in 0.2 ml. of fluid decreased gradually, but while using the solution with less NaCl the effect of the oxytocin was enhanced and became satisfactorily uniform.

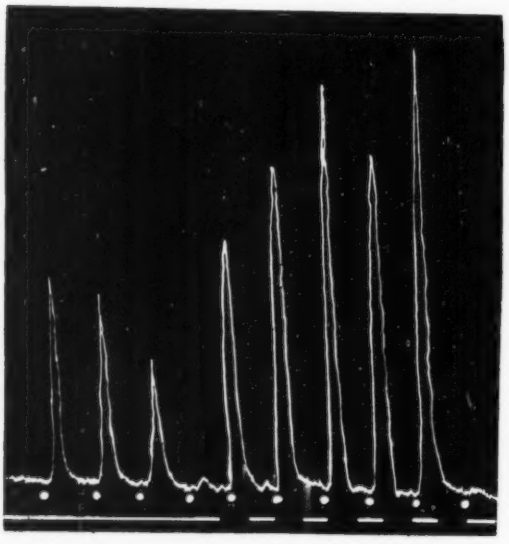

FIG. 1.—Effect of changing the concentration of sodium chloride on oxytocin induced contractions of a superfused rat uterus. Temperature of superfusion fluid, 33°; applications at 3 min. intervals. Solid line, 0.9 g./100 ml. of sodium chloride. Broken line, 0.4 g./100 ml. of sodium chloride. **1-3**: B.P. solution: 0.5 mU./ml. oxytocin. **4**: B.P. solution: no oxytocin. **5-9**: "Low sodium chloride" solution: 0.5 mU./ml. oxytocin. **10**: "Low sodium chloride" solution: no oxytocin.

Extraction of Oxytocin from Rat Blood.—Known amounts of oxytocin were added to rat plasma (4.0 and 8.0 mU./ml.) and extracted using the technique described above. In 5 experiments, the recovery of oxytocin was between 66 and 90% of the added amounts. The recovery of oxytocin added to whole rat blood in 8 experiments was between 78 and 104% of the amounts added. The mean recovery of oxytocin in all experiments with plasma and whole blood was $87 \pm 2.7\%$ (S.E. of means; $n=13$). In 2 of the experiments in which oxytocin was added to whole blood, the mixture was incubated for 10 min. at 37° before centrifugation and extraction. All the oxytocin was recovered from the plasma. This suggests that during the period of incubation oxytocin was not taken up by the blood cells. When precipitating plasma proteins, it was essential to add acetone to plasma, for with the reverse procedure less than 10% of the added oxytocin was recovered. Collection of the blood in chilled polyethylene tubes and strict adherence to the recommended time and speed for centrifugation were necessary, for, if the blood was centrifuged for less than 30 min. or collected in glass tubes, oxytocic activity which resisted treatment with sodium thioglycollate was present in the extracts. The most concentrated extracts of plasma were such that 1 ml. of the extract was equivalent to 1 ml. of plasma. The sensitivity of the uteri to oxytocin varied considerably and usually the lowest effective concentration was about 200 μU. oxytocin/ml. of standard solution, but occasionally very sensitive uteri were encountered with which as little as 20 μU./ml. of plasma could have been detected. Extracts of plasma from arterial blood from rats under ether anaesthesia seldom contained detectable oxytocic activity.

Effect of Haemorrhage on Oxytocic and Antidiuretic Activities in Blood.—As it has been shown that there is increased release of antidiuretic hormone from the neurohypophysis of anaesthetized rats during haemorrhage and that the concentration of the hormone in blood increases with the volume of blood withdrawn (Ginsburg and Heller, 1953c; Ginsburg and Brown, 1956), it seemed important to know whether, under similar conditions, the release of oxytocin was also increased.

The experiments were based on those described by Ginsburg and Brown (1956). Blood samples

containing the venous outflow from the cerebral circulation were withdrawn at 4 min. intervals from a cannulated external jugular vein in rats anaesthetized with ether. At the same time equal volumes of rat blood were returned to the animal by a femoral vein so that no change in blood volume should occur during collection of the sample. During each interval between the collection of such blood samples, more blood (0.5 ml./100 g.) was withdrawn without simultaneous return of blood, and thus the volume of blood lost by the animal increased regularly up to a total deficit of 3.0 ml./100 g. To obtain in each experiment sufficient material to determine both oxytocic and antidiuretic activities, blood samples were taken under similar conditions from two rats and the plasmas of corresponding samples were pooled.

The mean results of 4 experiments are shown in Fig. 2. No oxytocic activity could be detected in plasma, with blood deficits up to and including 1.5 ml./100 g., and in these instances the open rectangles in Fig. 2 give the mean concentrations at the limits of detectability. Antidiuretic activity in blood was raised as the blood loss increased. In blood taken when the deficit was 1.5 ml. blood/100 g. the antidiuretic activity was about 30 times that of control samples, while the oxytocic activity was still less than 0.4 mU./ml. plasma.

· *Inactivation of Oxytocin by Plasma* in vitro.—Before investigating the disappearance of oxytocin from the circulation *in vivo*, it was necessary to determine whether there was enzymatic inactivation of oxytocin in rat plasma as in plasma of pregnant

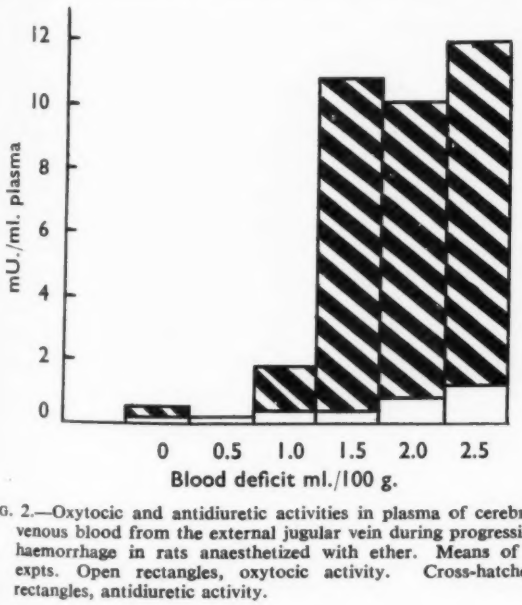

FIG. 2.—Oxytocic and antidiuretic activities in plasma of cerebral venous blood from the external jugular vein during progressive haemorrhage in rats anaesthetized with ether. Means of 4 expts. Open rectangles, oxytocic activity. Cross-hatched rectangles, antidiuretic activity.

TABLE I

INACTIVATION OF OXYTOCIN DURING INCUBATION WITH PLASMA AT 37° FOR 20 AND 40 MIN.

40 mU. of oxytocin was added to each ml. of plasma. Means ± S.E. are given. The numerals in parentheses indicate the number of experiments made.

Plasma	% Inactivation	
	20 min. Incubation	40 min. Incubation
Rat: in oestrus	11·6±4·8 (6)	8·3±4·4 (6)
,, pregnant	13·5±3·4 (5)	10·0±8·8 (6)
,, lactating	12·9±4·7 (6)	9·2±3·1 (6)
Human: 1 day prepartum	90·6±3·3 (3)	96·6±1·1 (3)
0·9% NaCl soln. ..	—	5·9±3·7 (7)

women (Page, 1946; Hawker, 1955). Table I shows the effect of incubating oxytocin at 37° for 20 and 40 min. in the presence of plasma from normal female rats in oestrus, rats in the third week of pregnancy, lactating rats in the second week postpartum, and a human subject one day before delivery. After incubation in plasma from the pregnant woman, the oxytocin was almost completely inactivated, but the loss of activity after incubation with the rat plasmas was only 6.3 to 13%, an amount which can be reasonably attributed to losses during extraction.

The Disappearance of Injected Oxytocin from the Circulation in Rats.—Oxytocin (200 mU./100 g.) was injected intravenously into male rats anaesthetized with ether and given heparin. Blood samples were collected from a cannulated carotid artery at intervals after the injection. A control blood sample (1.0 ml.) was taken about 5 min. before injecting oxytocin. The total volume of all the blood samples from an animal was as much as 4.5 ml., and, to limit the blood deficit to 2 ml. or less, 2.5 ml. of blood from another rat was injected intravenously immediately after withdrawal of the control blood sample.

Fig. 3 summarizes the results: each point is the mean concentration found in 3 to 7 experiments. When the oxytocin concentration in plasma is plotted on a linear scale (Fig. 3*a*), the points fall on an exponential curve; when the oxytocin concentration is plotted on a logarithmic scale (Fig. 3*b*) the fit to a straight line is very good apart from one point. The mean concentration (±S.E.) in plasma fell from 20.6±2.85 mU./ml. (n=7) 1 min. after injection to 1.0±0.09 mU./ml. (n=3) 6 min. later. On the assumption of an exponential relationship between oxytocin concentration and time after injection, the mean half-life was 1.65±0.13 min. (n=7).

The Disappearance of Oxytocin from the Circulation in Nephrectomized Rats.—Experiments

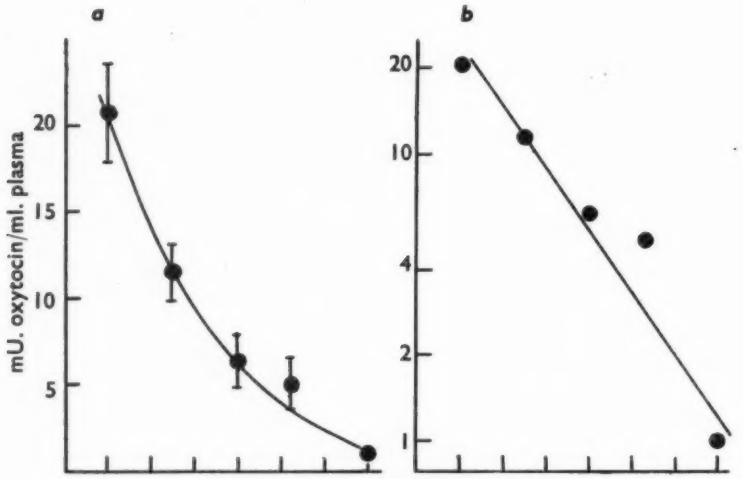

FIG. 3.—Concentrations of oxytocin in arterial plasma in intact male rats anaesthetized with ether following intravenous injection of 200 mU. of oxytocin/100 g. Each point is the mean of 3 to 7 experiments. *a*, linear; and *b*, log plots.

similar to those described in the previous section were performed on rats in which both kidneys had been removed, and in sham operated animals in which either the kidneys were exposed but not handled, or the kidneys were handled, ligatures were passed round the renal vessels and the perirenal fat was removed. Oxytocin (200 mU./100 g. of body weight) was injected intravenously 15 to 20 min. after completion of the operation.

The rate of disappearance of oxytocin from the circulation was not affected by sham operation when the kidneys had not been handled (half-life= 1.61 ± 0.11 min., n=4), but in animals subjected to the more severe sham operation the half-life was 2.73 ± 0.34 min. (n=6). It seems likely that the capacity of the kidneys to remove oxytocin from the circulation was not affected by their exposure but was depressed by handling, and in all subsequent operations in the abdomen care was taken to avoid handling the kidneys.

Nephrectomy retarded the disappearance of oxytocin from the circulation and the half-life was 2.95 ± 0.2 min. (n=6). The difference between this and the mean half-life in intact animals (1.65 min.) indicated the contribution of the kidneys to the disappearance of oxytocin from the circulation in intact animals.

Effect of Excluding the Splanchnic Vascular Area in Rats with and without Kidneys.—The circulation in the splanchnic vascular area was excluded by ligating the coeliac superior mesenteric arteries, dividing the ascending colon and left colic artery between ligatures, and ligating the portal vein. The satisfactory arrest of the circulation in the splanchnic vascular area was demonstrated at the end of each experiment by the bloodless appearance of the intestines despite the ligation of the portal vein, and by the absence of bleeding when the liver was cut.

15 to 20 min. after ligating the blood vessels, oxytocin (200 mU./100 g.) was injected intravenously and the concentrations of oxytocin were determined in plasma or blood withdrawn at intervals up to 7 min. after the injection. The rate of disappearance of oxytocin from the circulation was slightly less than in intact animals, the mean half-life for 4 experiments being 2.12 ± 0.20 min. compared with 1.65 min. in intact rats; this difference is not statistically significant ($0.1 > P > 0.05$). Also, exclusion of the splanchnic vascular area in acutely nephrectomized animals did not significantly alter the half-life compared with that found in animals nephrectomized only ($P > 0.05$). Thus, unlike the kidneys, the organs of the splanchnic vascular area do not affect the rate of disappearance of oxytocin from the circulation during the first 7 min. after injection.

In other experiments blood was collected during 26 min. after the injection of oxytocin. In animals which were only nephrectomized the concentration of oxytocin in plasma fell continuously; 14 min. after injection the mean concentration in plasma was less than 0.4 mU./ml. (Fig. 4). In animals in which the circulation of the splanchnic vascular area had been excluded in addition to nephrectomy, the concentration of oxytocin fell during the 7 min. immediately after injection as rapidly as in animals which were nephrectomized only, but thereafter there was little or no further change in oxytocin concentration in plasma.

This constant plasma oxytocic activity suggests that, after removal of the kidneys and exclusion of the circulation from the splanchnic vascular area, no organ or tissue remained in which oxytocin could be rapidly destroyed or inactivated. The continuous fall of the concentration of oxytocin in plasma in animals which were nephrectomized only must therefore be due to the uptake of oxytocin by the organs of the splanchnic vascular area. However, this is not rapid enough to affect materially the rate

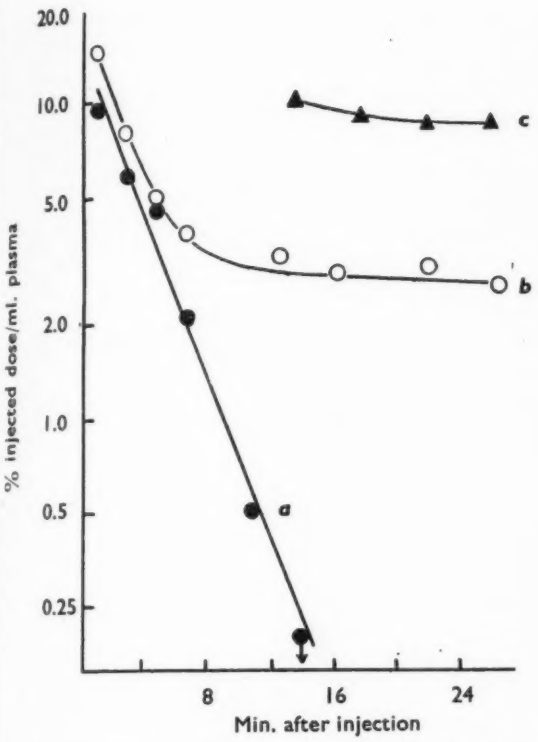

FIG. 4.—Concentrations of oxytocin in arterial plasma following intravenous injection of 200 mU./100 g. of oxytocin. *a*, nephrectomized rats; *b*, nephrectomized rats without splanchnic circulation; *c*, the concentration of inulin in arterial blood of nephrectomized rats without splanchnic circulation after intravenous injection of 40 mg. of inulin/100 g. Each point is the mean of 4 to 8 experiments.

at which oxytocin disappears from plasma during the first 7 min. after the injection.

Inulin Space in Nephrectomized Rats with Ligated Splanchnic Vascular Bed.—The inulin space was measured in nephrectomized rats with the vessels supplying the vascular splanchnic bed ligated. Inulin (40 mg./100 g. of body weight) was injected intravenously and blood samples were withdrawn at intervals up to 26 min. After injection the plasma concentration of inulin fell and became constant after 18 to 22 min. This concentration corresponded to a volume of distribution of 11.8 ± 0.45 ml./100 g. of body weight (n=7). The volume of distribution for oxytocin, obtained from the equilibrium oxytocin concentration in plasma in nephrectomized rats without splanchnic circulation, was 43.1 ±4.2 ml./100 g. (Fig. 4).

Disappearance of Injected Oxytocin from the Circulation in Female Rats.—The rate of disappearance of oxytocin after intravenous injection into normal female rats in oestrus was similar to that found in male rats (mean half-life=1.73 ± 0.10 min.; n=5). In pregnant rats 14 to 20 days after mating, the mean half-life for oxytocin was 2.01 ±0.08 min. (n=5) which is not significantly different from that found in oestrus animals ($P>0.05$). In lactating rats 3 to 14 days after parturition, the disappearance of injected oxytocin was significantly faster than in non-lactating animals, the mean half-life being 1.19 ±0.06 min. (n=6, $P<0.001$). Non-lactating females after

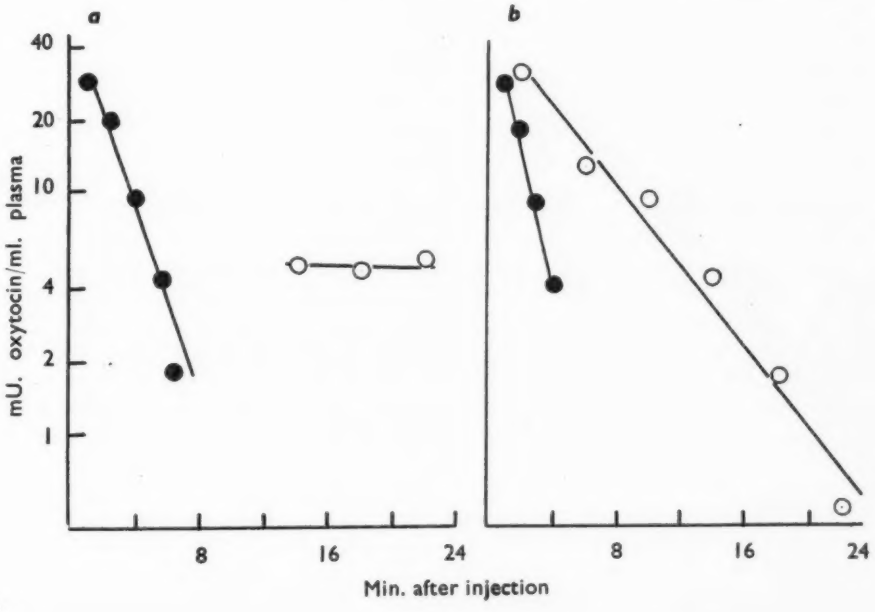

FIG. 5.—Effect of nephrectomy and exclusion of the splanchnic circulation on the disappearance of injected oxytocin from the circulation in: *a*, oestrus non-lactating rats; *b*, lactating rats. Each point is the mean of 3 to 6 experiments. ●——●: intact animals. O——O: nephrectomized and without splanchnic circulation.

bilateral nephrectomy with exclusion of the circulation in the splanchnic vascular area gave similar results to male rats (Fig. 5), there being no significant change in the concentration of oxytocin in plasma from 14 to 26 min. after the injection. On the other hand, after bilateral nephrectomy and exclusion of the splanchnic vascular bed in lactating animals, a constant concentration was not established and the oxytocin in plasma fell continuously and the plasma contained less than 0.2 mU./ml. 26 min. after the injection. In non-lactating females, the concentration was 5.2 ± 0.5 mU./ml. at this time.

Discussion

In male and non-lactating female rats the rapid rate of disappearance of injected oxytocin from the circulation is the resultant of three components and in lactating females there are four. Oxytocin disappeared most rapidly in rats with both kidneys and circulation to the splanchnic vascular area intact and with lactating mammary glands. When these organs or tissues were absent or excluded from the circulation, the concentration of oxytocin in plasma fell during the first 10 min. after injection and thereafter did not change greatly during the experiment. From this it may be concluded that no organ or tissue remained in which oxytocin was bound, destroyed, excreted, or in any other way irreversibly withdrawn from the exchangeable pool of oxytocin.

The volume of distribution for oxytocin under these conditions, calculated from the concentration of oxytocin in plasma when equilibrium was established, was three to four times greater than that found for inulin under the same conditions. Thus during the first 10 min. after injection in animals without kidneys and circulation in the splanchnic vascular bed, the fall in plasma concentration of oxytocin was due to equilibration of oxytocin in extra- and intra-cellular fluids. The fall in concentration was exponential during the first 7 min. with a mean half-life of 2.81 min. compared with 1.19 min. in intact lactating animals and 1.65 min. in intact males. Confirmation of the extensive penetration of oxytocin into body fluids was sought in experiments where oxytocic activity was assayed in extracts of skeletal muscle taken from animals after injection of oxytocin. No conclusion could be drawn from these experiments because, even when no oxytocin was injected, the extracts contained an unidentified oxytocic substance which, like oxytocin, was inactivated by treatment with sodium thioglycollate.

Although generalized penetration of oxytocin into extra- and intra-cellular fluids is possible and may be rapid, preferential uptake by the kidneys, organs of the splanchnic vascular area and the lactating mammary gland determines the fate of oxytocin in intact animals. The experiments of Heller and Lederis (1957) suggest that oxytocin may be bound to plasma proteins, but this is not incompatible with rapid diffusion into extravascular spaces if the formation of the complex is reversible.

Of the three special sites of uptake, the least important is the splanchnic vascular area. The best evidence for the participation of the splanchnic vascular area is the effect of excluding this area from the circulation of nephrectomized animals. In animals nephrectomized only, oxytocin disappeared continuously and no oxytocic activity was detected in blood 14 min. after injection. After excluding the splanchnic vascular area in nephrectomized animals there was no appreciable fall in oxytocin concentration between 10 and 26 min. after the injection. The mean concentration of oxytocin in plasma when equilibrium was reached was at least ten times greater than that found in plasma of rats with the splanchnic circulation intact 14 min. after injection. However, the rate of disappearance of oxytocin was not significantly affected by exclusion of the splanchnic circulation in animals with kidneys.

The kidneys play a more significant role in the removal of oxytocin from the circulation than do the organs of the splanchnic vascular area. Nephrectomy slowed the disappearance of oxytocin, the mean half-life increasing from 1.65 to 2.85 min. Chaudhury and Walker (1957) have obtained a similar result in rabbits. The present experiments give no indication of the ultimate fate of oxytocin taken up by the kidneys, but Larson (1939) has shown that up to 30% of oxytocin injected into cats and dogs can be recovered from urine within 2 hr. of injection.

In lactating animals the disappearance of oxytocin from the circulation was significantly faster than in males or non-lactating females. When the kidneys were removed and organs of the splanchnic vascular area were excluded from the circulation equilibration did not occur, and hence it was concluded that in lactating animals there is an additional organ or tissue in which preferential uptake of oxytocin takes place. This cannot be attributed to the appearance in blood of a system which inactivates oxytocin. It is most probable that the mammary gland is involved. The uterus, which is in a hypertrophied state, could also be implicated, but in non-lactating rats in oestrus

preferential uptake was restricted to the kidneys and the splanchnic vascular area, as in intact males. Sawyer (1954) found increased inactivation of oxytocin by ground-up uteri taken from pregnant rats after 14 to 21 days' gestation. In our experiments using rats at the same stage of pregnancy, the rate of disappearance of oxytocin from the circulation was similar to that in non-pregnant animals. More critical tests could have been devised to seek preferential uptake of oxytocin by the pregnant uterus, but these would have involved extensive operations in the abdomen which might have affected the uterus.

The half-life of oxytocin in the circulation has been determined in rabbits (Chaudhury and Walker, 1957), and can be calculated for cats from the results of Jones and Schlapp (1936). The values are: rat, 1.65 min., rabbit, 3.3 min., and cat, 8.5 min. The inverse relationship between the half-life and the size of the animal is not surprising, since circulation time will be a determinant of the rate of disappearance with a substance which disappears rapidly.

Although the hormones of the neurohypophysis occur in the gland in roughly equal proportions in terms of international units, there is evidence of a considerable preponderance of oxytocin over vasopressin in the secretion from the stimulated gland. The experimental evidence has usually been obtained by recording simultaneously the effects of stimuli on diuresis and uterine motility (Harris, 1948; Cross, 1951; Abrahams and Pickford, 1954), but it has been confirmed by estimation of oxytocic and antidiuretic potencies in blood (Chaudhury and Walker, 1958; Bisset, Lee, and Bromwich, 1956). Our results (preponderance of antidiuretic over oxytocic activity in cerebral venous blood collected during stimulation of the neurohypophysis by progressive haemorrhage) confirm that the hormones are not secreted in the same proportions as they occur in the gland, but unlike previous findings the results show that oxytocin is not always in excess.

The method described in this paper for extracting oxytocin from blood has the merits of simplicity and consistently high recoveries of added oxytocin. The use of acetone for preparing posterior pituitary powder does not immediately suggest that the oxytocin would remain in solution after acetone precipitation of plasma proteins, although Dudley (1923) and Heller and Lederis (1959) observed that some oxytocic activity was present in acetone extracts of posterior pituitary glands. When the extraction method was applied to plasma containing added vasopressin, recoveries were not satisfactory (never being greater than 60%) as might be expected since vasopressin is generally less soluble in organic solvents than oxytocin (Acher and Fromageot, 1955).

We are grateful to Professor G. G. Lennon for making available blood from a pregnant woman and to Mr. M. Ireland for technical assistance.

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ACTIONS OF SCORPION VENOM ON SKELETAL MUSCLE

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A protein-like constituent of scorpion venom produced effects on the skeletal muscle fibre resembling those of citrate, lack of calcium or veratrine. These effects could be diminished by the addition of calcium. They do not appear to be due to the formation of a non-ionized complex with calcium.

A notable feature of the experimental poisoning of laboratory animals with scorpion venom is the occurrence of irregular, frequent twitches and tetanic contractions of skeletal muscle. Wilson (1904 1921; cited by Rohayem, 1953) found that venom had a direct stimulating action on skeletal muscle, and that it caused a block of neuromuscular transmission which he considered to be unlike the block produced by curare.

The experimental work described in this paper was undertaken to investigate these findings more fully.

METHODS

The methods of obtaining, storing, and redissolving the venom have been described previously (Adam and Weiss, 1958). In all the experiments described the venom used was that of *Leiurus quinquestriatus* and all doses quoted refer to dried, whole venom from a pooled sample. The venoms of *Buthotus minax* and *Parabuthus hunteri* were also investigated and found to have similar action on the isolated muscle preparations.

Isolated Toad Sartorius Muscle Preparation.—The sartorius muscle of *Bufo regularis* was dissected out with the motor nerve cut at the entrance into the muscle, and put into a 1 ml. bath containing Ringer solution at room temperature (25°±4°). The composition of the Ringer solution (mM.) was: NaCl 116, KCl 2.0, $CaCl_2$ 1.8, $NaHCO_3$ 3.0. During the experiments the whole content of the bath was replaced by the relevant test solutions. When it was necessary to alter the concentration of calcium, the isotonicity was maintained by changing the concentration of NaCl. The contractions were recorded on a smoked paper using a spring-loaded lever with an amplitude magnification of 10.

Isolated Rat Diaphragm Preparation.—The method was that of Bülbring (1946), except that Krebs solution of the following composition was used (mM.): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, dextrose 11. The muscle was suspended in a 60 ml. bath at 37°. The *p*H remained between 7.2 and 7.3 when a mixture of 95% O_2 and 5% CO_2 was bubbled through the bath. In the experiments with an altered calcium concentration, isotonicity was maintained by changing the concentration of NaCl. Electrical stimulation was effected by an electronic stimulator delivering rectangular pulses through platinum electrodes at the rate of 0.2/sec. The preparation was stimulated either through the phrenic nerve or directly, in which case tubocurarine (0.2 μg./ml.) was added to the bath.

Isolated Toad Cardiac Muscle Preparation.—A strip of cardiac muscle approximately 1.5×1.5×15 mm. was cut from the ventricle and suspended in Ringer solution of the same composition as that used for the sartorius muscle, in a 1 ml. bath at room temperature (25°±4°). The strip was electrically stimulated by rectangular pulses at 0.2/sec. through two platinum electrodes.

Electrophoresis of Venom.—Electrophoresis of venom was carried out on Whatman No. 31 paper in 0.25 N-acetic acid, *p*H 2.6, at 0.25 mA./cm., or in borate buffer, *p*H 8.6, at 0.6 mA./cm. The paper was then cut longitudinally and one half of it treated with 0.2% (w/v) ninhydrin in acetone containing 10% (w/v) acetic acid (Jepson and Stevens, 1953), and subsequently heated at 90°. When the paper was viewed in ultra-violet light, 5-hydroxytryptamine as well as constituents giving the usual ninhydrin reaction (Adam and Weiss, 1958) were demonstrated. The other half of the strip was cut into the fractions indicated by the bands on the stained half, and these were eluted overnight in Ringer or Krebs solution at 4°. Other dyeing procedures used were the azocarmine method (Turba and Enenkel, 1950) and the amidoschwarz method (Grassmann and Hannig, 1950). Eluates were obtained as above.

RESULTS

Isolated Toad Sartorius Muscle.—The immersion of a sartorius muscle in a solution of venom (10 μg./ml.) in Ringer produced after a delay of 2 to 3 sec., a moderately rapid contracture upon which, at the peak of the amplitude, frequent short irregular twitches were often superimposed. The

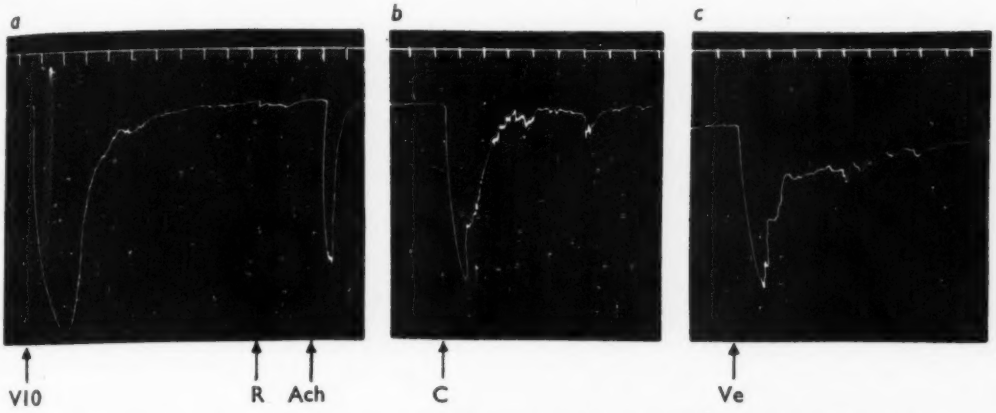

FIG. 1.—Comparison of effects of venom, veratrine, and citrate on the isolated toad sartorius muscle, isotonic recording. *a*: venom 10 μg./ml. (V10). R, refilled with fresh venom at same concentration. Ach, 2 μg./ml. of acetylcholine. *b*: C, sodium citrate 2.5 mM. *c*: Veratrine 0.2 mg./ml. (Ve). Time, 30 sec.

increased tone and the twitching continued for about 25 to 35 sec. Subsequently the tone fell slowly and irregularly, and the amplitude and frequency of the twitches diminished. Within 3 to 5 min. after the initial exposure to the venom, the muscle, still in the venom solution, had usually regained its original tone, but small, irregular twitches could be seen for periods up to 20 min. Draining of the bath and immediate refilling with a fresh solution of venom in the previous concentration did not produce a contracture, but other stimuli, such as acetylcholine or electrical stimulation, retained their effectiveness (Fig. 1*a*). Muscles which had been in contact with solutions of venom for more than about 30 sec. did not completely regain their original responsiveness to venom even if they were washed 3 to 4 times and allowed to remain in normal Ringer solution for as long as 30 min. Repeated exposure to venom at short intervals (1 min.), with two intermediate washings in Ringer, resulted in rapid tachyphylaxis; but after less than 30 sec. contact with venom, muscles regained their original responsiveness almost completely when washed and allowed to remain in normal Ringer for longer than 7 to 8 min. (Fig. 2).

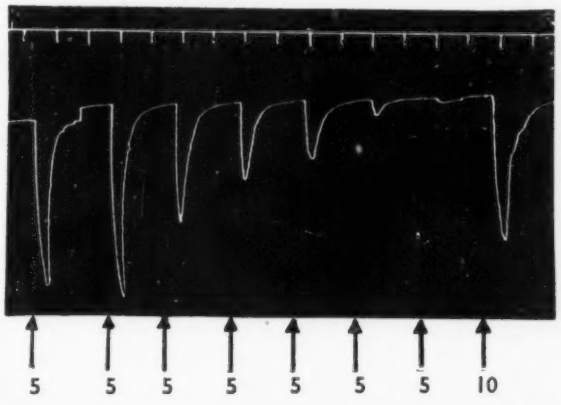

FIG. 2.—Isolated toad sartorius muscle. 5: Venom 5 μg./ml., 10: Venom 10 μg./ml., each in contact with muscle for 15 sec. Between first two doses, 8 min. in Ringer solution free of venom. Intervals of 1 min. between subsequent doses. Time, 30 sec.

The exposure of muscles to higher concentrations of venom produced more rapid contractures with shorter delay periods and greater amplitudes. At concentrations above about 16 μg./ml. of venom maximal twitches were often superimposed on the contracture: lower concentrations of venom produced smaller contractures after a longer delay (Fig. 3). Venom concentrations

FIG. 3.—Isolated toad sartorius muscle. Effects of 0.5, 1, 2, 4, 8, 16, and 32 μg./ml. of venom, each in contact with muscle for 15 sec. The numerals indicate the amounts of venom. Time, 30 sec.

below 2.0 μg./ml. did not produce consistent responses, but concentrations of 0.5 or 1.0 μg. venom/ml. sometimes produced small irregular twitches, without any increase in the muscle tone after a delay of 30 sec. to several minutes. After the addition of sufficient tubocurarine to repress completely the response to acetylcholine (2 μg./ml.), the muscles did not show any alteration in their responses to venom.

Isolated Rat Diaphragm.—The effects of immersing the isolated rat diaphragm in Krebs solution containing venom did not differ in principle from those on the toad sartorius muscle, but the contracture was better maintained (Fig. 4). Smaller

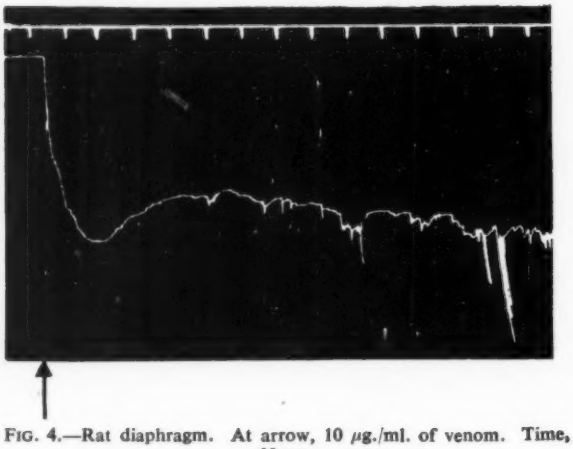

FIG. 4.—Rat diaphragm. At arrow, 10 μg./ml. of venom. Time, 30 sec.

concentrations of venom, as low as 1.0 μg./ml., caused reproducible contractures. After repeated contact at short intervals, tachyphylaxis to venom appeared in the same way as with the sartorius muscle. The effects of venom could be reversed by washing if the time of exposure to the venom was kept short, not exceeding 1 min. With higher concentrations of venom (10, 15, and 20 μg./ml.), the responses had shorter delays and greater amplitudes. After the addition of tubocurarine (2 μg./ml.) to the bath, the response to venom was unaltered.

Modification by Venom of the Effects of Electrical Stimulation of Rat Diaphragm.—When venom was applied to the bath during direct electrical stimulation of the preparation at 5/min., the response was modified as follows: (1) The amplitude of the electrically-induced contractions was increased at first, and later diminished (Fig. 5*a*). This increase in amplitude was superimposed on the contracture previously described. It was seen only when the electrical stimuli applied were submaximal, suggest-

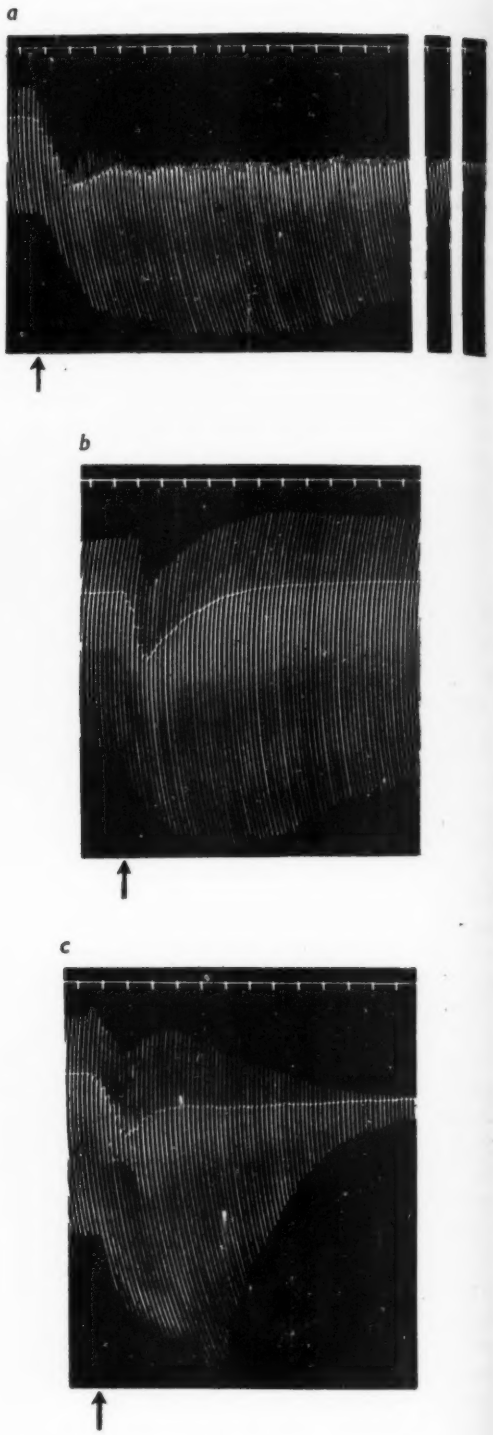

FIG. 5.—Rat diaphragm. Direct submaximal stimulation. *a*: 5 μg./ml. of venom at arrow. Between the last two records 15 min. elapsed. *b*: 10 mM. sodium citrate at arrow. *c*: 35 μg./ml. of veratrine at arrow. Time, 30 sec.

ing that the effect was due to a lowering of the threshold rather than to an increased contractility. (2) The rate of relaxation was decreased. This appeared to be connected with small spontaneous twitches induced by the venom which were very obvious when watching the irregular return of the writing lever after each induced contraction. A slower rate of relaxation was also indicated by a disappearance of the rebound overshoot of the recording lever.

Similar effects were produced by venom when the rat diaphragm was stimulated indirectly through its phrenic nerve.

Comparison of the Effects of Venom, Citrate, and Veratrine.—The effects of venom on the striated muscle preparations bore some resemblance to those of calcium-complexing agents and to those of veratrine. We compared therefore the effects of venom, sodium citrate, and veratrine on the toad sartorius muscle and the rat diaphragm.

On the toad sartorius muscle, the three agents produced a qualitatively similar effect, consisting of a contracture followed by a slow irregular relaxation, accompanied by twitching, whilst the agents remained in the bath (Figs. 1*a*, *b*, *c*). On the electrically-stimulated rat diaphragm the three agents gave a contracture and an initial increase in amplitude of the twitches, followed by a decrease, but the time relation of this effect differed. When doses of the three agents which induced approximately equal contractures were used, the subsequent depression set in most rapidly with veratrine and least rapidly with citrate, the effect of venom being intermediate. A further difference was that the slowing of relaxation and the twitching, which are characteristic features of the action of venom, were absent when veratrine or citrate was used (Figs. 5*a*, *b*, *c*).

When veratrine was applied during the depressant phase of venom action, the muscles were found to be more sensitive, and a contracture could be produced with only about a quarter of the veratrine dose normally required to produce the same effect on a fresh muscle. Muscles did not become more sensitive to venom during contact with veratrine.

Effects of Changes in Calcium Concentration.—Since citrate lowers the level of ionized calcium, and since displacement of calcium from binding sites at excitable membranes has been suggested as an explanation of the action of veratrine (Gordon and Welsh, 1948), the effects of changes in the calcium concentration in the bath fluid on venom action were investigated.

On both the sartorius muscle and diaphragm, high concentrations of calcium diminished the effects of venom. Doubling the calcium concentration diminished the venom effect on the sartorius muscle, and this diminution increased with time (Fig. 6). A four-fold increase in the calcium concentration completely abolished the effect of 10 μg./ml. of venom. On the rat diaphragm, venom antagonized the depression by high calcium concentrations of the electrically-induced contractions. Fig. 7 shows the depression produced by a four-fold increase in the calcium concentrations, and the

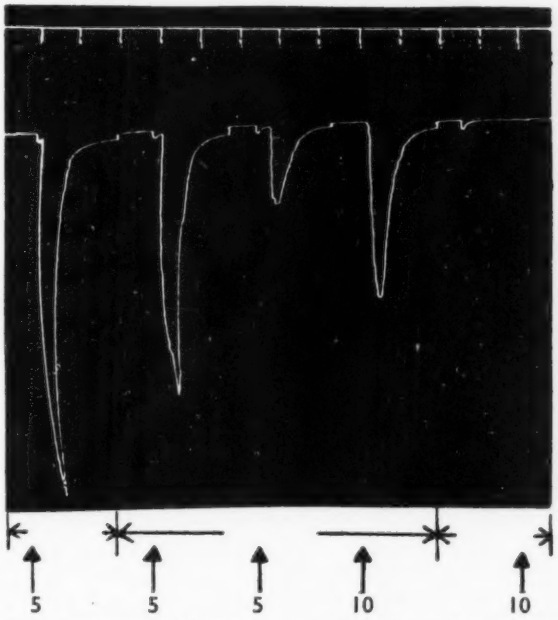

FIG. 6.—Isolated toad sartorius muscle. Effect of venom in presence of increasing calcium concentration. 10 min. elapsed between each dose of venom. **5**: 5 μg./ml. of venom. **10**: 10 μg./ml. of venom. The calcium concentration was doubled at each pair of horizontal arrows. Original concentration 1.8 mM. Time, 30 sec.

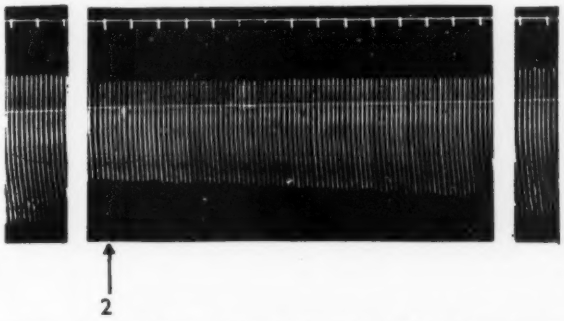

FIG. 7.—Rat diaphragm. Direct submaximal stimulation. Effect of 1.8 mM. calcium in the first record. In the second and third records, the calcium concentration was increased four-fold. Between the first and second record 1 min. elapsed, and between the second and third 6 min. elapsed. **2**: 2 μg./ml. of venom. Time, 30 sec.

restoration by venom of the amplitude of the contractions.

In Ringer solution containing no calcium, the sensitivity of the toad sartorius muscle to venom was increased (Fig. 8): the sensitivity of the electrically-stimulated rat diaphragm to venom increased so that 0.5 μg./ml. of venom caused a distinct contracture. Further, if a diaphragm had been in contact with a concentration of venom not normally producing a contracture (say, 0.5 μg./ml.), the subsequent immersion of the preparation in Krebs solution containing neither calcium nor venom gave a contracture (Fig. 9).

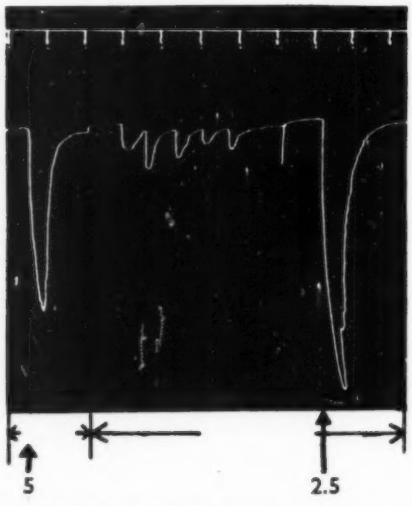

FIG. 8.—Isolated toad sartorius muscle. Effect of calcium-free Ringer solution on that of venom. **5**: 5 μg./ml. of venom. **2.5**: 2.5 μg./ml. of venom. Original calcium concentration 1.8 mM. between left-hand horizontal arrows. Changed to calcium-free Ringer between right-hand pair of horizontal arrows. Time, 30 sec.

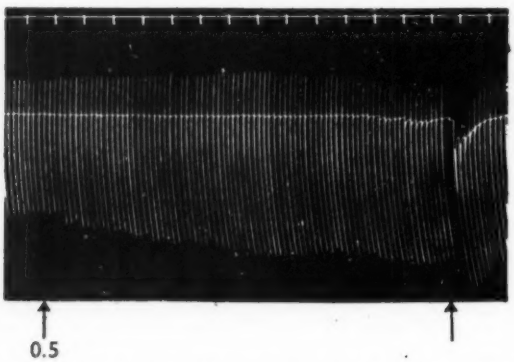

FIG. 9.—Rat diaphragm. Direct submaximal stimulation. Effect of immersion in calcium-free Krebs at second arrow after previous contact with a small dose of venom at first arrow. **0.5**: 0.5 μg./ml. of venom in Ringer solution of normal composition. Time, 30 sec.

When toad cardiac muscle was placed in calcium-free Ringer solution, the threshold to electrical stimulation was promptly lowered and, within a short time, the amplitude of the contractions decreased. Restoration of the normal calcium-concentration gave a rapid reversal. The addition of citrate 5 mM./l. to Ringer solution had a similar effect on the threshold and on the amplitude of the contractions as the absence of calcium from the bath fluid and was also fully reversible. Venom in concentrations of 5 to 60 μg./ml. markedly lowered the threshold to electrical stimulation, but neither affected the amplitude of the contractions nor produced a contracture.

Nature of the Active Principle in Venom.—When kept in a desiccator for several months the crude, dried venom did not lose any activity as measured by contractions which it produced in the sartorius muscle and rat diaphragm preparations. In aqueous solution (1 mg./ml.) at 4°, activity was maintained for 1 to 2 weeks. Venom solutions (20 μg./ml.), heated in a boiling water bath at *p*H 7 for 10 min., lost 80% of activity on sartorius muscle and rat diaphragm. Complete loss of activity occurred after heating for 30 min. in a boiling water bath at *p*H 7. Incubation of venom (10 μg./ml.) with trypsin or chymotrypsin (100 μg./ml.) for 30 min. at 37° at *p*H 7.4 resulted in a complete loss of activity on the muscle preparations. The active constituent did not dialyse detectably through cellophane in 24 or 48 hr. at 4° against 5 volumes of Ringer solution or distilled water changed 4 times. The crude venom could be split by electrophoresis into several fractions giving a violet reaction with ninhydrin. With azocarmine and amidoschwarz, only two distinct bands were seen. Eluates of the slower band produced the same effects as crude venom on the various types of muscle described. This fraction moved towards the cathode at both *p*H 2.6 and 8.6. Eluates of the other fractions had no effect on the muscle preparations. It was concluded that the principle active on the muscle preparations is probably protein in nature.

DISCUSSION

The effects of venom, large doses of veratrine, and substances like citrate, which bind calcium ions, were in some respects similar. They all produced a contracture, an initial increase in the amplitude of electrically-induced muscle contractions, and spontaneous twitching. These similarities could be explained by the assumption that the stabilizing function of calcium at the muscle membrane is interfered with by the active constituent of venom.

The subsequent gradual decrease in amplitude of electrically-induced contractions may well be due to fatigue caused by the continual minute twitches and increased tone which were main features of the effect of venom.

Increased concentrations of calcium lessened the effects of venom, and lowered concentrations enhanced them, findings which are in accord with the above hypothesis. When the response of the diaphragm to electrical stimulation was depressed by high concentrations of calcium, it could be restored by adding venom to the bath fluid. Further, in both rat diaphragm and toad sartorius muscle, a contracture could be produced by a combination of a dose of venom, normally subthreshold, and calcium-free Ringer solution.

Two different mechanisms of interference by venom with calcium can be envisaged. Firstly, it may have an action like that of citrate, lowering the concentration of ionized calcium in the surrounding medium by forming an undissociated complex. The active constituent of venom is probably a protein and several proteins are known to form complexes with calcium (Brink, 1954). However, a consideration of the effective doses of venom (2 to 5 μg./ml. of dried crude venom) applied in a calcium concentration of 1.8 mM. indicates that an improbably large number of calcium ions would have to be taken up by each molecule of the venom substance to produce the effects of lack of ionized calcium from the medium. Further, if complex-formation is involved, venom would be expected to produce other effects which resemble calcium-lack, but a concentration of 100 μg./ml. of venom did not interfere with the clotting of blood (Smith, 1958, personal communication). On cardiac muscle the only similarity between the effects of venom and of calcium-lack was a marked decrease in the threshold to electrical stimulation. The lack of effect of venom on the strength of contraction of cardiac muscle would indicate that it did not act at those sites which control the strength of contraction, and for which calcium and sodium compete (Niedergerke and Luttgau, 1957).

The alternative mechanism is a displacement of calcium by venom from the sites that bind calcium at the muscle fibre membrane. A similar explanation of the mode of action of veratrine has been proposed tentatively by Gordon and Welsh, 1948; see also Shanes, 1958.

Differences between the effects of venom and of veratrine have already been mentioned. They consisted mainly in a more rapid depression of the amplitude of electrically-induced contractions and a lack of effect on relaxation by veratrine as compared with venom. In these respects, the effect of veratrine was less like that of calcium-depletion than was the effect of venom. Indeed, various observations, such as the lack of repetitive after-discharge when the whole muscle fibre was exposed to a uniform concentration of veratrine (Burns, Frank, and Salmoiraghi, 1955), and the depression of excitability that occurred after the first depolarization during contact with veratrine (Frank, 1958), are not consistent with the concept that the action of veratrine is due entirely to lack of calcium. Increased excitability and repetitive after-discharges are known to occur when calcium is lacking (Brink, 1954; Shanes, 1958), and it is possible that the mechanical effects of venom observed are associated with similar electrical events. It is obviously necessary to obtain direct measurements of resting potentials and negative after-potentials under the influence of venom before further conclusions on its mode of action can be reached.

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A COMPARATIVE STUDY OF THE EFFECTS OF DRUGS ON THE AROUSAL SYSTEM OF THE BRAIN

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The effects of twelve drugs, eleven of which are reported to be tranquillizers, have been studied in relation to thresholds for arousal produced both by direct stimulation of the brain stem reticular formation and also by afferent nerve stimulation. The drugs can be grouped according to whether (a) like chlorpromazine they produce a slight rise in thresholds for brain stem stimulation and block sensory-induced arousal, (b) cause dissociation between behaviour and electrical activity of the brain, (c) have no effects on thresholds for brain stem stimulation and only slight effects on afferent nerve-induced arousal or (d) have no effect on arousal responses at all.

The effects of twelve different drugs on arousal responses have been investigated. Most of the drugs chosen for the present study have been classed as tranquillizers on the basis of their clinical effects although there are differing reports regarding the clinical efficacy of some of them. It is, however, not our intention to discuss this question here.

Much of the early experimental work with chlorpromazine suggested an action at the level of the reticular activating system of the brain stem (Terzian, 1952; Longo, von Berger, and Bovet, 1954; Hiebel, Bonvallet, and Dell, 1954) and further investigations (Bradley and Hance, 1957) confirmed this. The drug blocked the behavioural and electroencephalographic effects of arousal stimuli, both in the conscious unrestrained animal and in acute preparations (*encéphale isolé*) and also blocked the excitant action of amphetamine, which is itself thought to act at the brain stem level (Bradley and Elkes, 1957). A more detailed study of the effects of drugs on arousal responses (Bradley and Key, 1958) demonstrated that chlorpromazine had little depressant action on the brain stem activating system itself but produced a selective blocking of the afferent influences on this system. This led to the hypothesis that chlorpromazine might act by depressing the collateral inflow into the reticular formation from the afferent pathways. So far no evidence has been produced which suggests that this hypothesis is wrong and further studies support it (Bradley, 1957; Key and Bradley, 1958). On the other hand we have not attempted to suggest that this particular central action of chlorpromazine is solely responsible for its clinical properties or that it is the only action of this drug on the central nervous system.

The tranquillizers form a group which, for almost all investigated properties, is so heterogeneous that we thought it might be worth while to examine the effects of a number of these compounds upon the arousal system. In this way it should be possible to see how far the hypothesis which has been elaborated to explain the action of chlorpromazine in neurophysiological terms might be extended to include other tranquillizers.

METHODS

Experiments were carried out on 56 adult cats, all of which were *encéphale isolé* preparations (Bremer, 1936). The animals were prepared under ether anaesthesia using a technique which has been described by Bradley and Key (1958). Twelve to sixteen cortical electrodes (Bradley and Elkes, 1953) were inserted through small burr holes in the skull over both cerebral hemispheres, and an earthing electrode was put in the midline over the frontal sinus.

A concentric, bipolar stimulating electrode, with the points separated by about 1 mm., was orientated in the brain stem reticular formation by means of a Horsley-Clarke stereotactic instrument, using co-ordinates obtained from an atlas of the cat brain. When the stimulating electrode was in the correct position it was fixed with dental cement and the animal was released from the stereotactic instrument. Local anaesthetic (procaine) was infiltrated in the skin and muscle over the top of the head and at least 1 hr. was allowed to elapse following the removal of ether before any measurements of thresholds were made.

The electrocorticogram was recorded on a 4-channel Ediswan portable pen recorder and stimuli were delivered from an electronic stimulator remotely controlled. The stimulus lasted for a period of 10 sec. and consisted of rectangular pulses of 1 msec. duration at a frequency of 300 cycles/sec. The thresholds were determined by gradually increasing the applied voltage in steps, each lasting for 10 sec., starting from 0.1 V. The electrocorticogram was recorded continuously and, when the threshold for a behavioural response was reached (recognized by opening of the eyes and contraction of the nictitating membrane), the record was examined to see at what voltage it had been activated. This was taken as the threshold for electrocortical arousal.

In some experiments the threshold for arousal produced by sensory stimulation was also determined. An auditory stimulus of constant frequency was used, obtained from a loudspeaker placed at a fixed distance from the head of the cat. The procedure for determining the arousal threshold was the same as that for stimulation of the brain stem; the voltage which was applied to the loudspeaker and which caused arousal was taken as a measure of the threshold. In these experiments the thresholds for click responses at the auditory cortex were also determined.

The drugs studied were: chlorpromazine, promazine, acepromazine, imipramine, hydroxyzine, benactyzine, hyoscine, meprobamate, azacyclonal, reserpine, deserpidine, and rescinnamine. With the exception of the last three, all the drugs were injected intravenously in incremental doses and the arousal thresholds checked twice after each injection, usually after 10 and 20 min. Reserpine, deserpidine, and rescinnamine appear to have a delayed action and a single dose of these drugs was injected and the thresholds checked at 30 min. intervals.

At the end of each experiment the animal was killed and the brain perfused with formal-saline with the stimulating electrode still in place. The position of the tip could be subsequently determined by histological examination.

Results

The *encéphale isolé* preparation shows fluctuating states of wakefulness and sleep. These two states are easily distinguished by the appearance of the head and by the electrical activity of the brain. In the waking and apparently "alert" state, movements of the ears, eyes, and occasionally the jaws and vibrissae may be seen together with a pupillary response to light. The electrical activity of the cortex is fast and of low voltage, similar to that in the intact animal in the alert state (Rheinberger and Jasper, 1937; Bradley and Elkes, 1957). When the animal is in the drowsy or "sleeping" state the nictitating membrane is relaxed, the eyes are rotated upwards and the pupil is fixed and constricted, whilst the electrocorticogram is dominated by slow waves at 1 to 4 cycles/sec. and spindle activity at 6 to 12 cycles/sec. This correspondence between the electrical activity and the behavioural state in these preparations is always maintained, although individual variations in the pattern of "sleep activity" can often be recorded.

Activation of the electrocorticogram and behavioural arousal can still be evoked in *encéphale isolé* preparations by certain types of sensory stimuli, since the brain is capable of receiving afferent impulses from the intact cranial nerves. In the present series of experiments, electrical stimulation of the brain stem reticular formation produced almost identical responses, except that the period of activation following the stimulus was usually longer. The transition from the "sleeping" to the "alert" state was abrupt and well marked.

Control experiments were carried out to determine the viability of the preparations and the stability of the arousal thresholds. Thresholds for behavioural arousal produced by stimulation of the reticular formation, determined over periods of 2 to 4 hr., showed very little variation. The first sign of deterioration in the preparation was usually a slow falling of blood pressure. Following this, the behavioural response became less marked and artefacts appeared in the electrocorticogram which made the determination of the arousal threshold more difficult.

Experiments were also undertaken to determine the stability of arousal responses produced by an auditory stimulus of constant frequency. Thresholds were first determined and then checked every. 20 min. over 3 to 4 hr. In all preparations habituation to the stimulus developed very rapidly. The duration of the period of activation in the electrocorticogram progressively decreased and at the fourth trial behavioural arousal was indicated by only a slight movement of the ears. By the fifth to sixth trial, stronger stimuli were needed to produce arousal, but even then the responses were weak. In the eighth and subsequent trials the stimulus failed to produce any alteration in the electrocorticogram or in the behaviour of the animal even when the intensity of the stimulus was increased considerably above control levels (Fig. 2*a*). If, at this stage, the frequency of the auditory stimulus was changed, or another of marked qualitative difference was presented, arousal was immediately obtained. Similarly, pinching the ear or blowing on the face produced arousal.

Chlorpromazine

The effects of this drug on the thresholds for arousal produced by stimulation of the brain stem

have already been reported (Bradley and Key, 1958). It was found in fifteen experiments that doses of 2.0 to 4.0 mg./kg. of body weight caused a slight rise in thresholds for both electrocortical and behavioural arousal (Fig. 1*a*). Larger doses caused no further change in thresholds and, above 10 mg./kg., artefacts appeared in the electrocorticogram. These were thought to be due to changes in blood pressure induced by such large doses of chlorpromazine. An interesting feature of about one-third of these experiments was that very small doses of chlorpromazine (0.1 to 0.5 mg./kg.) caused a slight fall in the behavioural and electrocorticographic thresholds, but with a total dose of 0.8 mg./kg. the thresholds returned to their original levels. This effect was not observed with any of the other drugs tested.

In contrast to these results, the effect of chlorpromazine on arousal responses produced by sensory stimulation was quite marked. Thresholds for behavioural arousal and electrocorticographic activation produced by auditory stimulation remained unaltered with doses of chlorpromazine between 0.1 and 0.3 mg./kg., but when the dose was increased from 0.5 to 1.8 mg./kg. both thresholds showed a progressive rise (Fig. 2*b*). Moreover, the arousal responses themselves became less marked. For example, after 0.8 mg./kg. the period of activation in the electrocorticogram was shorter and did not usually outlast the period of stimulation, whilst behavioural arousal was indicated by only a slight movement of the ears and a transient and partial opening of the eyes. When the total dose was increased to 2.0 to 4.0 mg./kg., auditory-evoked arousal was blocked completely, irrespective of the frequency or quality of the auditory stimulus. At the same time, the preparation became unresponsive to other types of sensory stimulation, although pain (caused by pinching the ears) still produced some response. The threshold for click responses recorded at the auditory cortex remained unchanged throughout the experiment.

In two experiments, (±)-amphetamine (4.0 mg./kg.) was given after chlorpromazine, but this drug was without effect on the electrical activity of the cortex or on the arousal thresholds, and the behavioural state induced by chlorpromazine was not modified. These observations confirm previous studies (Bradley and Hance, 1957).

Promazine

The effect of promazine on arousal responses produced by stimulation of the reticular formation was studied in three experiments. The drug was injected in doses of 0.1 to 20.0 mg./kg. No fall in threshold was observed with small doses (0.1 to 0.3 mg./kg.) although there was transient rise and fall in blood pressure similar to that seen with chlorpromazine. When the total dose was increased to 1 to 2 mg./kg., a slight rise in both behavioural and electrocortical arousal thresholds occurred (Fig. 1*b*). Increasing the dose further, however, did not produce any additional change although high doses (5 to 10 mg./kg.) caused large blood pressure fluctuations which affected the electrical activity of the cortex.

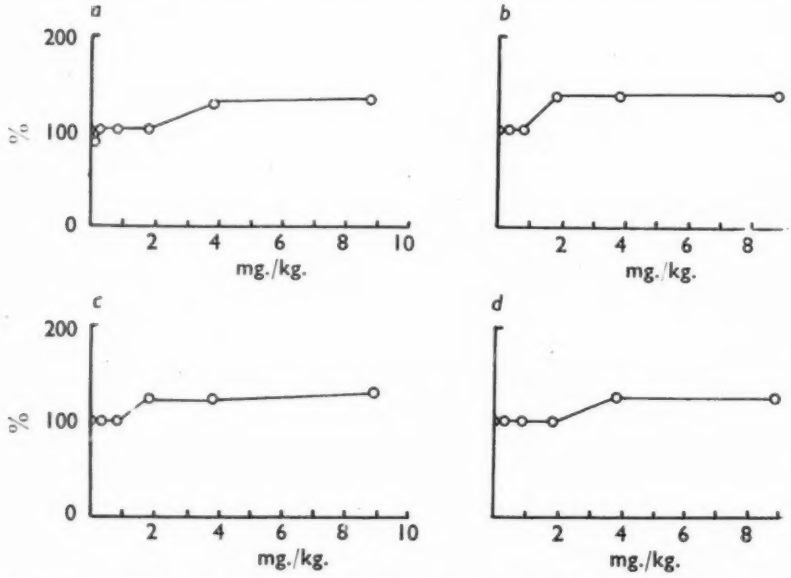

FIG. 1.—The effects of four drugs on thresholds for arousal produced by stimulation of the brain stem reticular formation. The mean % change in threshold for all experiments has been plotted against dose. *a*, Chlorpromazine; *b*, promazine; *c*, acepromazine; and *d*, hydroxyzine.

The effects of promazine on auditory-induced arousal were great and similar in many ways to those of chlorpromazine. With doses of 0.3 to 2.0 mg./kg., the preparations became less easy to disturb and, following 1.0 to 2.0 mg./kg., very intense auditory stimulation produced only a transient change in the electrocorticogram. Similarly, visual and tactile stimuli which had previously evoked arousal responses now failed to do so. Increasing the dose above 2.0 mg./kg. usually blocked arousal induced by afferent stimuli completely although pain stimuli sometimes still produced a

transient activation of the electrocorticogram and an alerting of behaviour.

Physostigmine and atropine given with promazine produced their characteristic effects on the electrical activity of the cortex without affecting the behaviour of the animal. Amphetamine, in large doses (4 to 6 mg./kg.), brought about a slight increase in the responsiveness of the preparation to afferent nerve stimulation, but the full effects of amphetamine on the electrical activity of the cortex and the behaviour of the animal were never observed. Thus, amphetamine appeared to block only partially the effects of promazine.

Acepromazine

Acepromazine was injected in three experiments in doses of 0.1 to 20.0 mg./kg. Qualitatively and quantitatively the effects on arousal responses alone or with atropine, physostigmine, and amphetamine did not appear to be different from those of promazine. After 1.0 to 2.0 mg./kg. of acepromazine there was a slight increase in the thresholds for arousal produced by stimulation of the brain stem reticular formation (Fig. 1*c*). Concomitantly, the preparations became unresponsive to visual, auditory, and tactile stimulation.

Hydroxyzine

Hydroxyzine (10 mg./kg.) had very little effect on the electrical activity of the brain in *encéphale isolé* preparations. There was usually an increase in the amount of slow activity, but this was always associated with increased drowsiness of the animal.

Although periods of spontaneous electrocorticographic activation and behavioural alertness were noted, they were less frequent than in the control period before the drug. Furthermore, physostigmine and atropine still produced their characteristic effects on electrical activity when given with hydroxyzine, but they did not change the behavioural state of the animal. These effects closely paralleled those when chlorpromazine was used with these two drugs. However, whereas chlorpromazine completely blocked the effects of amphetamine both on the electrical activity of the brain and on behaviour, the latter drug still produced some effect when it was given after hydroxyzine, although there was never complete alerting. Thus, hydroxyzine like promazine appeared to block only partially the effects of amphetamine.

The effect of hydroxyzine on arousal responses produced by stimulation of the reticular formation

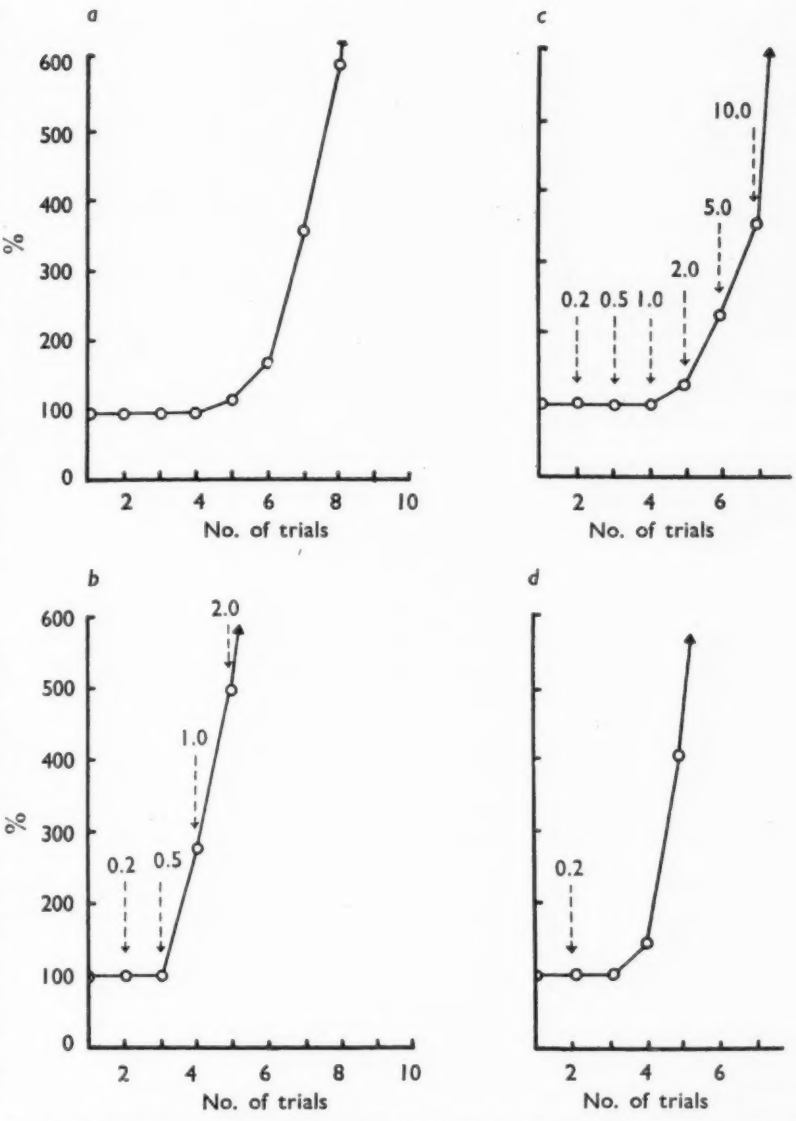

FIG. 2.—The effects of drugs and habituation on thresholds for arousal produced by afferent nerve (auditory) stimulation. The mean % change in threshold for all experiments has been plotted against the number of trials. The injections of drugs are indicated by the arrows, with doses in mg./kg. *a*, Habituation; *b*, chlorpromazine; *c*, azacyclonal; and *d*, reserpine.

was studied in four experiments. The drug was injected intravenously in incremental doses from 0.1 to 8.8 mg./kg. and thresholds for arousal were found to be unchanged until a total dose of 2 to 4 mg./kg. was reached. With this dose both electrocortical and behavioural arousal thresholds showed a slight but consistent rise (Fig. 1*d*). No further increase in the threshold was observed with larger doses, although it was sometimes difficult to establish the electrocorticographic threshold owing to the low blood pressure brought about by the large doses of this drug.

The effects of hydroxyzine on arousal responses induced by sensory stimuli were also studied and they were found to be similar in many ways to those observed with chlorpromazine. Doses of 0.8 to 1.8 mg./kg. caused a progressive rise in the thresholds for both the electrocorticographic and behavioural arousal, and when a total dose of 2 to 4 mg./kg. was reached arousal responses to auditory stimuli were completely blocked, irrespective of the frequency. Sometimes an intense auditory stimulus, qualitatively different from the previous one, would produce an effect when first applied, but this soon disappeared. Similarly, visual and tactile stimuli failed to evoke an arousal although the response to pain was little altered. The threshold for click responses recorded at the auditory cortex remained unchanged even when the sensory-induced arousal responses were blocked.

Benactyzine

Benactyzine was given in three experiments in doses of 0.1 to 4 0 mg./kg. With 0.2 mg./kg. or less, no effects on the thresholds for arousal produced by stimulation of the brain stem were seen, but as the dose was increased above 0.5 mg./kg., large amplitude slow waves, similar to those after atropine, appeared in the electrocorticogram. At the same time the threshold for electrocortical arousal was raised, reaching a maximum with doses between 3.0 and 4.0 mg./kg. (Fig. 3*a*). The effect of benactyzine on the threshold for electrocorticographic arousal was not associated with any corresponding change in that for behaviour and the threshold/dose curves for electrocorticogram and behavioural arousal showed a wide divergence. A similar dissociation was also noted in the threshold/dose curve for arousal produced by afferent (auditory) stimulation. After 0.8 to 2.0 mg./kg. of benactyzine activation of the electrocorticogram by auditory, visual or tactile stimuli was blocked completely but there was no observable effect on the behavioural alerting response. Moreover, amphetamine given in conjunction with benactyzine always caused

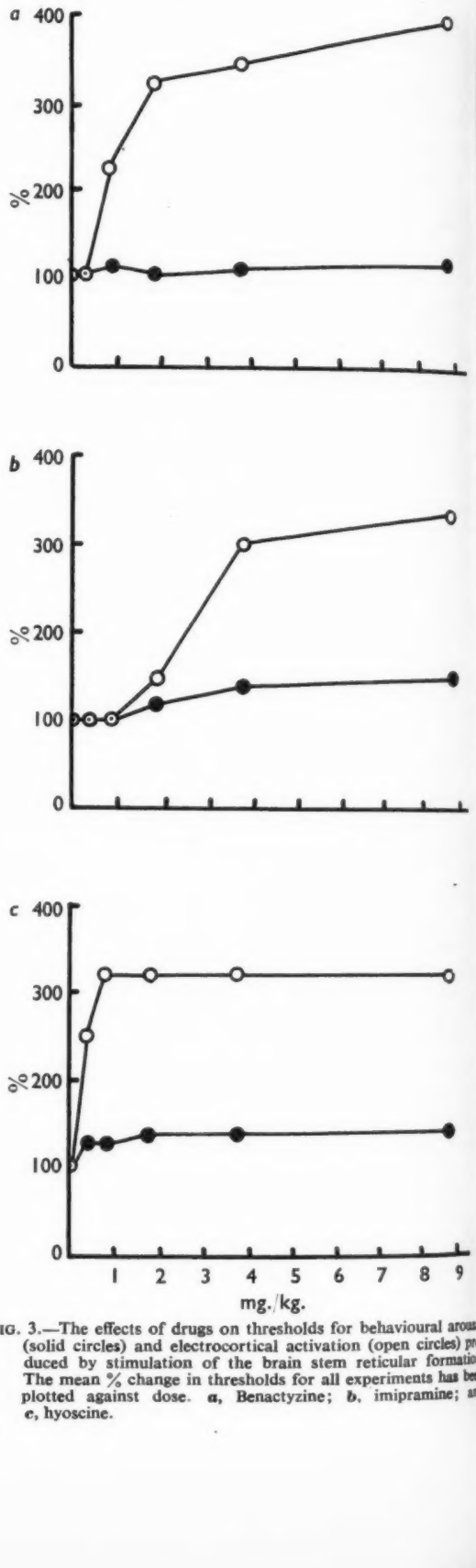

FIG. 3.—The effects of drugs on thresholds for behavioural arousal (solid circles) and electrocortical activation (open circles) produced by stimulation of the brain stem reticular formation. The mean % change in thresholds for all experiments has been plotted against dose. *a*, Benactyzine; *b*, imipramine; and *c*, hyoscine.

alerting of behaviour without affecting the electrical activity of the cortex. Physostigmine desynchronized the electrocorticogram without producing behavioural alerting.

Imipramine

Imipramine is an aminodibenzyl derivative which bears certain structural resemblances to chlorpromazine. For this reason and the fact that recent studies have shown that this drug may be of use in clinical psychiatry in the treatment of depression, it was included in the present study.

The drug was given in doses of 0.1 to 20 mg./kg. at intervals of 20 min. With 0.1 mg./kg. or less no effects on arousal thresholds were seen, but larger doses caused a progressive rise in the threshold for electrocortical arousal produced by stimulation of the reticular formation (Fig. 3*b*), and slow activity, similar to that seen after atropine, appeared in the electrocorticogram. There was also a slight rise in the behavioural threshold after 2.0 to 4.0 mg./kg., but the increase was never as great as that for electrocorticographic arousal and the threshold/dose response curves for this drug always showed a marked divergence.

Electrocortical activation produced by sensory stimulation was blocked completely after 0.1 to 2.0 mg./kg. Behaviourally, however, the animal still responded to afferent nerve stimulation, provided the intensity was above control levels.

When amphetamine was given after imipramine the sedation produced by the latter drug was completely antagonized. Increasing doses of amphetamine from 0.5 to 3.0 mg./kg. caused the behavioural arousal threshold to decrease until the animal became completely alert. At the same time the electrical activity of the cortex showed a decrease in the amount of slow activity, but a marked discrepancy between the thresholds for electrocortical and behavioural arousal still remained.

Physostigmine following imipramine abolished the slow waves induced in the electrical activity of the cortex by the latter drug but in no way affected the behavioural state of the preparation or modified the threshold for behavioural arousal.

Hyoscine

The effects of hyoscine on arousal responses have been reported in another paper (Bradley and Key, 1958). It was included here because the effects it produced were in many ways similar to those of benactyzine and imipramine. Less than 0.03 mg./kg. was without effect, but larger doses induced slow wave activity in the electrocorticogram and caused the threshold for electrocorticographic arousal produced by brain stem stimulation to increase progressively (Fig. 3*c*). The maximum effect appeared with doses of 0.9 to 1.0 mg./kg. and a further increase in the dose caused no additional change. There was a slight increase in the threshold for behavioural arousal with doses of 1.0 mg./kg. The threshold/dose response curves showed a marked divergence, however. If physostigmine was injected in doses from 0.1 to 1.0 mg./kg. the threshold for electrocorticographic arousal, which had been raised by hyoscine, was progressively lowered to zero as the slow activity was abolished and replaced by low amplitude, fast activity. The threshold for behavioural arousal usually fell to the control level or sometimes remained slightly raised. Amphetamine, however, always resulted in complete alerting of behaviour.

Reserpine

Since the thresholds for arousal responses of the *encéphale isolé* preparation are not stable for periods longer than 5 to 6 hr. and since reserpine appears to have a delayed action, incremental doses of this drug could not be given. Instead a large dose of 250 or 500 μg./kg. was injected intravenously and the arousal thresholds checked after each succeeding $\frac{1}{2}$ hr. over a $3\frac{1}{2}$ hr. period.

The effects of reserpine were slow to appear and no apparent change in the behavioural state of the animal was seen until 1 to $1\frac{1}{2}$ hr. after the injection. The threshold for arousal produced by stimulation of the reticular activating system remained unaltered but the preparation became less easy to disturb by sensory stimulation. Thresholds for behavioural and electrocortical arousal produced by the auditory stimulus rose progressively and after $2\frac{1}{2}$ to 3 hr. both responses were blocked completely (Fig. 2*d*). In contrast the threshold for click responses recorded at the auditory cortex remained unchanged and at this point stimulation of other sensory modalities still produced responses. In addition, arousal could be obtained if a different auditory stimulus was applied, but the intensity of the latter was sufficiently high to suggest that the threshold may have increased. The threshold/time response curves after the administration of reserpine may therefore represent the effect of the drug on sensory-induced arousal responses, together with the effect of habituation to the stimulus.

In some experiments following a dose of 500 μg./kg., the incidence of slow wave activity characteristic of the "drowsy" state decreased until the electrocorticogram consisted entirely of low amplitude, fast activity. This change was not reflected on the behavioural state of the animals and for most of the time they appeared to be asleep.

Rescinnamine and Deserpidine

These two drugs were given in six experiments in single doses of 250 μg./kg. or 500 μg./kg. Thresholds were determined every ½ hr. over a 3½ hr. period following the injection. Both deserpidine and rescinnamine appeared to exert effects similar to those of reserpine on responses tested in the *encéphale isolé*. Arousal responses produced by stimulation of the brain stem reticular formation remained unaltered throughout the experiment whilst there was a tendency with the higher dose (500 μg./kg.) for faster rhythms to appear in the electrocorticogram. This was not associated with any change in the behaviour of the animals, which continued to sleep for most of the time.

After 1 to 1½ hr. the preparations became a little less easy to disturb with auditory and visual stimuli although the response to pain remained unaltered. Thus, as long as the frequency of the stimulus was not constant, auditory stimulation continued to alert the animal behaviourally and produced activation of the electrocorticogram.

Meprobamate

In the present study three experiments were conducted with meprobamate in doses of 0.1 to 40 mg./kg. There was no change in the thresholds for arousal produced by stimulation of the reticular formation, and even with the highest doses no appreciable alterations were observed in the patterns of electrical activity of the cortex. In quantitative studies of the effects of meprobamate on sensory-induced arousal there was an increase in both the behavioural and electrocortical arousal thresholds until finally the animal failed to respond. This effect, however, was not dose-specific. Thus the blocking of sensory-induced arousal was probably due to habituation to the stimulus, since other stimuli (auditory, visual, or tactile) still produced arousal responses.

Azacyclonal

This drug was injected in doses of 0.1 to 25 mg./kg. in three experiments. As in the case of meprobamate no effect on the threshold for arousal produced by stimulation of the reticular formation was observed and the preparations continued to show periods of wakefulness and sleep. Even after 25 mg./kg. the patterns of electrical activity of the cortex remained unchanged, and behaviourally the animal showed no signs of sedation. Thresholds for arousal produced by auditory stimuli rose progressively during the experiment until eventually the animal failed to respond (Fig. 2*c*). This effect, as with meprobamate, was not dose-specific and, since the animal responded to other afferent stimuli, it could not be distinguished from habituation.

Discussion

The results show that the drugs studied can be classified into four groups according to their effect or lack of effect on arousal responses. *Group* 1: Drugs which caused slight depression of arousal responses produced by direct stimulation of the brain stem reticular formation and affected the electrocortical activity and behaviour in the same way; they also depressed thresholds for arousal produced by afferent nerve stimulation. *Group* 2: Drugs which increased arousal thresholds but at the same time produced a dissociation between behaviour and electrical activity. *Group* 3: Drugs which had no effect on arousal thresholds but which, in high doses, caused a change in the electrocorticogram towards the activation pattern. *Group* 4: Drugs with no detectable effects. The properties of the drugs in each of these groups will be discussed in more detail.

Group 1.—Chlorpromazine, promazine, acepromazine, and hydroxyzine all fall into this group. As can be seen in Fig. 1, they all increased the thresholds for arousal produced by stimulation of the brain stem reticular formation, but this rise was only slight and was never more than a 50% change. The rise was roughly the same for all four drugs but occurred with different doses. Thus with chlorpromazine and hydroxyzine the effect appeared with doses between 2.0 and 4.0 mg./kg. and with promazine and acepromazine between 1.0 and 2.0 mg./kg. With all four drugs the effect was not progressive and once it had occurred further increases in the dose caused no appreciable change. The threshold for behavioural arousal and electrocortical activation always changed together and there was no dissociation between the electrocortical activity and behaviour. No fall in the arousal threshold for brain stem stimulation, such as had often been seen with small doses of chlorpromazine, was observed with the other three drugs.

The effects of chlorpromazine on the threshold for arousal produced by sensory stimulation are shown in Fig. 2*b*. Promazine, acepromazine, and hydroxyzine caused similar effects on thresholds for sensory-induced arousal responses and doses which increased arousal thresholds for brain stem stimulation blocked arousal responses caused by visual, auditory, and tactile stimuli completely.

On the basis of evidence presented here, the mode of action of these drugs cannot be a direct depression of the arousal mechanism at the level of the

reticular activating system. On the other hand, the lack of effect of chlorpromazine on the electrical activity of the *cerveau isolé* preparation (Bradley and Hance, 1957) and the similarity between the effects exerted in the *encéphale isolé* and those seen in the intact animal led to the suggestion that chlorpromazine might exert its main action on some structure lying between the two planes of section in the acute preparations at a brain stem level. This suggestion is further supported by the results of experiments in which chlorpromazine was given with other drugs. Amphetamine, which is thought to act at the level of the brain stem (Bradley and Elkes, 1957), is antagonized by chlorpromazine (Bradley and Hance, 1957). This result has been confirmed in the present study, but whereas chlorpromazine completely blocked the effects of amphetamine the latter drug still produced some effect when it was given after promazine, acepromazine, or hydroxyzine, although there was never complete alerting. Thus, these drugs appeared to block the effects of amphetamine only partially. In the experiments in which the drugs of Group 1 were given with atropine and physostigmine, the latter drugs produced their usual effects on the electrical activity of the brain but did not modify the behavioural state of the animal. However, since it is unlikely that atropine and physostigmine act at the level of the brain stem (Bradley and Elkes, 1957), it might be expected that if the drugs of Group 1 do act at this level then the behavioural state of the animal would remain unaltered.

It has since been suggested, on the basis of the effect on afferent nerve-induced arousal responses, that chlorpromazine exerts an action which is related to the inflow of impulses from the ascending sensory pathways into the reticular formation (Bradley and Elkes, 1957; Bradley and Key, 1958). The similarity between the effects of chlorpromazine and those of promazine, acepromazine, and hydroxyzine on the thresholds for arousal produced by an auditory stimulus would indicate that the latter drugs act in a similar way to chlorpromazine. Thus the hypothesis put forward for the site of action of chlorpromazine can be extended to include the other three members of Group 1.

The pharmacological properties of the drugs in Group 1 lend further support to this suggestion. All of them exhibit some antispasmodic action against 5-hydroxytryptamine, histamine, and acetylcholine (Tripod, 1957), but probably more outstanding is the fact that, of all the drugs tested in the present series of experiments, chlorpromazine (Courvoisier, Fournel, Ducrot, Kolsky, and Koetschet, 1953), promazine (Tripod, 1957), acepromazine (Schmitt, Mercier, Aurousseau, Hallot, and Comoy, 1957) and hydroxyzine (Hutcheon, Scriabine, and Schrogie, 1957) are the ones which exhibit the most potent antiadrenaline properties, both at the periphery and centrally. Thus the hypothesis of a site of action in the brain stem reticular formation and the adrenergic properties of this system (Bonvallet, Dell, and Hiebel, 1954) would be consistent with current information on the properties of these drugs.

Group 2.—This group contains benactyzine and imipramine, for which a tranquillizing action has been reported, and we have also included hyoscine since its effects on arousal responses were similar. All caused a marked rise in the threshold for electrocortical activation produced by stimulation of the brain stem reticular formation and induced slow waves similar to those seen in sleep. Effects on the threshold for behavioural arousal were either absent (benactyzine) or only slight (imipramine and hyoscine) (Fig. 3). The drugs therefore caused a dissociation between the electrocorticogram and behaviour and in this respect they resembled atropine. None of these drugs antagonized the alerting effects of amphetamine as far as behaviour was concerned, nor did they block thresholds for behavioural arousal induced by afferent nerve stimulation.

It would appear that these drugs have little or no action on arousal mechanisms as far as behaviour is concerned and their effects on the electrical activity of the cortex are similar to those of atropine. It has been suggested that the action of atropine, unlike that of amphetamine, is not restricted to the brain stem reticular formation, but is probably exerted much more diffusely in producing changes in the electrocorticogram (Bradley and Elkes, 1957). The three drugs may have a similar action to atropine with which, in fact, they showed many other properties in common. For example, the administration of amphetamine after either benactyzine, imipramine, or hyoscine alerted the behaviour but in no way affected the electrical activity of the cortex, whereas physostigmine replaced the slow wave electrocortical pattern with low voltage fast activity but did not modify the behavioural state of the preparation. Moreover, in contrast to the drugs of Group 1, benactyzine, imipramine, and hyoscine are weakly anti-adrenaline (Tripod, 1957; Berger, 1957; and Lehmann, Cahn, and de Verteuil, 1958) and may, as benactyzine, even potentiate the pressor responses of adrenaline. However, all three drugs of this group show anti-acetylcholine properties and they appear

to be more effective in blocking the blood pressure and antispasmodic effects of acetylcholine than any of the drugs in the other groups.

Group 3.—This group includes reserpine and two related compounds, rescinnamine and deserpidine. The effects of all three drugs were the same. They caused no change in the threshold for arousal by stimulation of the brain stem although high doses produced a change in the electrocorticogram towards faster frequencies which was, however, unrelated to the behavioural state and usually the animals were asleep. The drugs appeared to block arousal by an auditory stimulus, but it was difficult to assess how much this was due to habituation to the stimulus (Fig. 2*a*) and how much it was due to the drug (Fig. 2*d*), since a novel stimulus usually had some effect. It is useful to compare this effect with that of chlorpromazine, which blocks the effects of all such stimuli, including those to which the animal had become conditioned (Key and Bradley, 1958). Thus reserpine, rescinnamine, and deserpidine, in contrast to chlorpromazine and other drugs of Group 1, appear to have no direct action on the reticular activating system of the brain stem nor do they block the effects of afferent nerve stimulation on this system but appear merely to modify these influences. The concept of an action at a cortical level, producing an increase in " cortical inhibition of diencephalic centres " (Schneider, Plummer, Earl, and Gaunt, 1955), would not conflict with this conclusion. In fact, it may offer a possible explanation of the slight increase in the threshold for sensory induced arousal seen in previous experiments (Key and Bradley, 1958) since the cortex is known to exert some influence upon conduction within the brain stem reticular formation (French, Hernandez-Peon and Livingston, 1955; Adey, Segundo, and Livingston, 1957). The precise pharmacological and biochemical mechanisms underlying such an inhibitory influence must at present remain an open question. 5-Hydroxytryptamine has been implicated in the central depressant action of reserpine (Woolley and Shaw, 1957), and it is interesting to note that, of all the rauwolfia alkaloids, only those which release 5-hydroxytryptamine are effective in psychiatric practice (Pletscher, Shore, and Brodie, 1956). The three compounds of Group 3 fall into the above category, but it must be remembered that these drugs also possess appreciable antispasmodic activity against adrenaline, acetylcholine, and histamine and have no effect against strychnine toxicity, while in contrast to Group 1 drugs they potentiate the pressor responses of adrenaline. It is likely, therefore, that reserpine, deserpidine, and rescinnamine have a common site and mode of action and that these are different from those of chlorpromazine and the other drugs in Group 1.

Group 4.—The two drugs of this group, meprobamate and azacyclonal, have been classified together for convenience. They are conspicuous in the present study not for what they did but for what they failed to do. Meprobamate, in doses (20 to 40 mg./kg.) which are said to produce tranquillization (Berger, 1954), had no effect on thresholds for arousal induced by direct stimulation of the reticular formation. The responses to afferent stimuli of all modalities were little changed, and even with the largest doses the patterns of electrical activity of the cortex remained unaltered. Similarly, azacyclonal produced no change in arousal thresholds nor did it alter the responses to visual, tactile or auditory stimuli unless the frequency of the latter was kept constant, resulting in habituation (Fig. 2*c*). It seems unlikely, therefore, that the site of action of these drugs is related to receptors situated at the level of the reticular activating system of the brain stem, unless the receptors are completely unrelated to the arousal system. The tranquillizing action of azacyclonal has been linked with inhibition of 5-hydroxytryptamine (Rinaldi, Rudy, and Himwich, 1955; Costa, 1956), whilst the central muscle relaxant properties of meprobamate may be implicated in the action of this drug. The pharmacological properties of meprobamate and azacyclonal show that Group 4 is a heterogeneous one. Meprobamate has little antispasmodic action against acetylcholine, histamine, 5-hydroxytryptamine, and adrenaline, whereas azacyclonal is said to inhibit contractions of isolated rat uteri induced by 5-hydroxytryptamine. However, neither drug releases 5-hydroxytryptamine and of the two only meprobamate is effective against strychnine convulsions.

All the drugs used in this study have been reported to be useful in the clinical treatment of psychiatric disturbances. It is therefore not possible to compare their effects on arousal responses with those of drugs having little or no clinical usefulness. Nor must we suggest at this stage that the electrophysiological effects produced by these drugs are related to the mode of action by which they change the mental states in man. We need to know much more about the mechanisms underlying mental disturbances before this can be attempted.

It is interesting to note that those drugs which have the most marked effect on arousal thresholds (Group 1) are used clinically to treat disturbed

psychiatric patients and therefore are presumably most effective in reducing psychomotor hyperactivity. It is for these drugs that the most satisfactory hypothesis regarding their mode of action has been produced, namely, that they depress the influence of afferent stimuli on the reticular formation at the brain stem level, and this hypothesis appears to cover all the drugs in this group. An inhibition of afferent stimuli could well account for some of the clinical effects of these drugs such as their tranquillizing action, potentiation of barbiturate anaesthesia, and anti-emetic effect. Azacyclonal and meprobamate showed no electrophysiological effects and there is some doubt or disagreement about their clinical effectiveness. It is difficult to account for the action of reserpine from its electrophysiological effects and we must be careful not to draw conclusions from observations of electrical activity unrelated to behaviour. Since the effects of this drug are so slow in appearing, we should perhaps look more closely at the biochemical mechanisms involved in its action.

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THE ANALYSIS OF DRUG-INDUCED TREMOR IN MICE

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An improved method of recording tremor in mice has been developed using a gramophone pick-up and a cathode ray oscillograph. Permanent records are made on 35 mm. film from which it is possible to determine qualitative differences in the tremor caused by various drugs. In addition, the severity of the tremor may be estimated quantitatively by a tremor index. The rate and variations in the rhythm and amplitude and in the tremor index have been determined for Tremorine (1,4-dipyrrolidin-1′-ylbut-2-yne), harmine, harmaline, 3-amino-1,1,3-triphenylpropan-1-ol and lysergic acid diethylamide. The incidence of side effects has also been noted.

Conventional methods of recording gross movements in small animals by means of a jiggle cage or work adder are not sufficiently precise to permit a critical analysis of tremor. The record obtained depends greatly on the type of apparatus used and, in addition, is likely to show movements arising from the resonance of the apparatus.

An improvement in technique was introduced by Moore, Sigg, and Schneider (1957), who implanted small permanent magnets subcutaneously in mice. The mouse was then surrounded by a wire coil and the changes in the induced current caused by tremor-producing drugs were recorded. The main disadvantage of the method is that the animals require preliminary operation. In our experience the animals are liable to die from sepsis, even if the magnets are first coated with Perspex and carefully sterilized by ultra-violet light.

The fidelity with which a gramophone pick-up will follow movement applied to the stylus has been exploited not only in recording motor activity (Larsen, 1955), shivering (Boyarsky and Stewart, 1957) and epileptic seizures (Essig and Flanary, 1957) in animals, but also tremor in man (Rohracher, 1946). In the experiments reported here we have attempted to characterize the tremor induced in mice by a variety of drugs and to express the severity of the degree of tremor by means of a tremor index.

METHOD

The mouse cage consisted of a transparent plastic soap box weighing about 30 g. (F. W. Woolworth Ltd.), the lid being perforated with ¼ in. holes to allow free access of air. The two halves were closed by two elastic bands. The box was attached to a gramophone pick-up (Model G.P. 31, Cosmocord Ltd.) by a short length of thin brass rod, one end of which was threaded and fixed to the lid by two nuts, the other end being inserted into the needle holder of the pick-up (Fig. 1). The output of the pick-up was fed directly into a cathode ray oscillograph (Cossor Model 1049) and the tracing photographed on 35 mm. film (Kodak R 55) with an oscillograph camera (Mk. II A, Cossor Instruments Ltd.). In some experiments, the output of the cathode ray oscillograph was capacitance-coupled to an amplifier driving a single channel direct writing pen recorder (Mk. 5, Henry Hughes and Sons Ltd.) and the record obtained on a continuous strip of

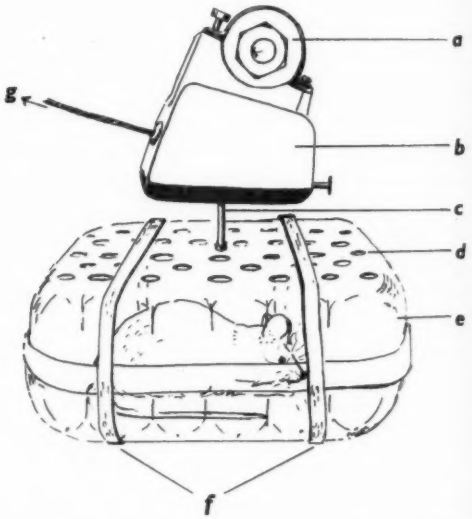

FIG. 1.—Soap-box pick-up assembly. *a*, supporting arm (viewed end on); *b*, pick-up; *c*, stylus; *d*, air holes; *e*, soap-box; *f*, elastic bands; *g*, screened lead to cathode-ray oscilloscope.

"Teledeltos" paper. The pick-up and soap box were mounted on a rigid slotted angle stand which was cushioned on thick sponge rubber to prevent vibrations being transmitted from the bench. Since the apparatus was microphonic, the cage and stand were placed under a telephone booth (Burgess "Accousti Booth") and the experiments conducted away from noise. The apparatus responded only to bodily movements of the mouse; movements attributable to respiration or heart beat did not appear. The frequency, wave-form and tremor index (see below) were obtained from the photographic records; the duration of tremor and the effect of anti-tremor drugs were obtained from the paper strip.

Albino mice weighing 18 to 30 g. of both sexes were used. Tremor was induced by intraperitoneal administration of the following drugs: Tremorine (1, 4-dipyrrolidin-1′-ylbut-2-yne) (30 mg./kg.), an amino alcohol (3-amino-1,1,3-triphenylpropan-1-ol) (30 mg./kg.), harmine (15 mg./kg.), harmaline (15 mg./kg.), lysergic acid diethylamide (4 mg./kg.), eserine (5 mg./kg.), and nicotine (5 mg./kg.).

Recording of the Tremor.—A mouse was placed in the soap box which was then fixed in position on the pick-up and the animal allowed to settle down for 10 to 20 min. During this time the spontaneous activity subsided almost completely and, when the mouse was quiet, control records were taken. The mouse was then removed, given an effective dose of one of the tremor producing drugs and replaced in the box. Records were then made for 10 sec. at intervals of 5 min. Some mice were treated with anti-tremor drugs and the results recorded in the same way.

Interpretation of the Records.—After processing, lengths of the film were bound between 16 in. strips of perspex and the tracing was examined under a five-fold magnification in a photographic enlarger. From the image cast on the screen, the frequency could be determined and an impression gained of the regularity of the rate and amplitude. The severity of the tremor was estimated by relating the distance traversed by the spot of light as a multiple of the straight line which the spot would have traced had no tremor taken place at all. This was done as follows: two parallel pencil lines were drawn on a piece of white cardboard 18 in. apart and the card was placed on the enlarger board so that the lines were intersected perpendicularly by the image of the light track. The length of the light track between these two lines was then measured to the nearest 0.1 in. by a sensitive opisometer. The length of the light track in inches divided by 18 gave the *Tremor Index*. For example, if the image of the light track as measured by the opisometer was 54 in., this divided by 18 would give a tremor index of 3.0.

Results

Untreated Mice

If an untreated mouse, or one given an injection of saline, was put in the box it showed the usual exploratory activity, cleaning movements and frequent alterations in position. Such spontaneous activity caused very irregular waves of small amplitude (Fig. 2, upper record). A few larger waves appeared in groups here and there and these were associated with cleaning movements. The frequency of the spontaneous movements was 22 to 25 cycles/sec. Left undisturbed, the mouse would settle down in about 10 to 20 min. The mice were allowed food and water *ad libitum* before the experiment since it was found that hungry mice took much longer to settle down. The tremor index of spontaneous activity varied between 1.30 and 1.51 when the mouse was first placed in the box and this decreased to 1.00 during the settling-down period.

Tremorine

Mice receiving Tremorine, 20 to 30 mg./kg. intraperitoneally, showed severe tremor, diminished normal motor activity, and profuse salivation, lachrymation and diarrhoea. The tremor was regular and continuous, and had a frequency of 14 to 18 cycles/sec. (Fig. 2, middle record). There was no marked variation in the amplitude of the tremor during the peak of the effect. The tremor index varied from 2.06 to 3.90 (Table I).

There was a latency of 2 to 5 min. before tremor appeared. The onset was gradual, fine tremor appearing first in the head and spreading later to the body and tail. In most animals the peak of the tremor occurred between 10 and 20 min. after giving the drug. The tremor persisted at rest and during movement. After about 30 to 45 min. the tremor became coarse and intermittent, resembling shivering. Some animals showed this coarse episodic tremor for as long as 4 hr.

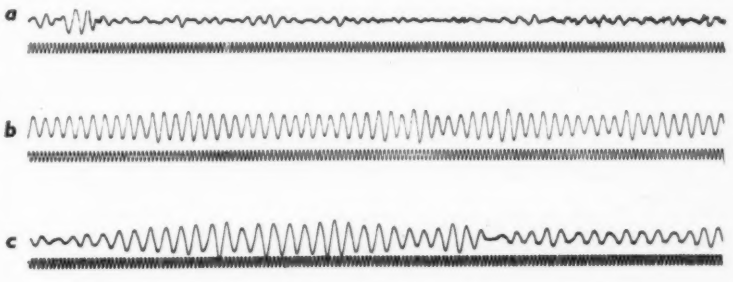

FIG. 2.—Cathode-ray oscillograph recording of (*a*) normal movement in the mouse, (*b*) tremor caused by Tremorine (30 mg./kg.) and (*c*) harmaline (15 mg./kg.). Time, 50 cycles/sec.

TABLE I

COMPARISON OF PATTERNS OF TREMOR INDUCED BY VARIOUS DRUGS IN MICE

n.r. indicates not recorded.

	Drugs						
	Tremorine	Harmine	Harmaline	Lysergic Acid Diethylamide	3-Amino-1,1,3-triphenyl-propan-1-ol	Eserine	Nicotine
Delay in onset (min.) ..	3–6	3–5	3–5	7–10	6–15	1–2	1–2
Duration (min.)	30–180+	15–40	20–40	15–30	30–120+	1–3	5–8
Cycles/sec.	14–18	14–16	14–16	18–20	15–17	n.r.	n.r.
Variability in amplitude	Slight	Slight	Slight	Moderate	Marked	,,	,,
Tremor index	2·06–3·90	2·20–2·38	2·1–2·5	1·97–2·3	2·25–3·4	,,	,,

Antagonism of Tremorine Tremor.—Blockus and Everett (1957) reported that atropine, hyoscine and other anti-parkinsonian drugs antagonized tremor induced by Tremorine. The effect of hyoscine on the tremor index was obtained as follows. A mouse was given Tremorine (30 mg./kg.) intraperitoneally and, during the peak of the tremor, received hyoscine hydrobromide (10 mg./kg.) intraperitoneally. The tremor ceased within 5 min. and normal behaviour returned in 15 to 30 min. The tremor index was lowered from 3.2 to 1.2, which is within normal limits.

Harmine and Harmaline

Harmine and harmaline are two alkaloids from the seeds of *Peganum harmala.* As early as 1895, Neuner and Tappeiner reported that these alkaloids could produce tremor in laboratory animals. The pharmacological properties of the two substances have been described by Gunn (1911, 1913).

Harmine produced tremor in mice after an injection of 10 to 20 mg./kg., intraperitoneally. The tremor appeared within 4 to 6 min. and lasted for 15 to 20 min.; the peak effect came between 8 and 15 min., at which time the tremor was continuous. The tremor was rather fine and generalized and was present both at rest and during movement. The animals showed the Straub tail phenomenon and hunched-back attitude but could walk with normal gait. The mice responded in an almost normal manner when handled. Reflexes were not exaggerated, there was no muscular weakness or lethargy and parasympathomimetic effects did not occur.

The pattern of tremor and the changes in behaviour induced by both harmine and harmaline were alike except that the intensity and duration of tremor were slightly greater with harmaline. Harmaline tremor appeared soon after the injection, lasted for 30 to 40 min. and was accompanied by slight piloerection. The tremor was regular and continuous although the amplitude waxed and waned (Fig. 2). The frequency of the tremor induced by both drugs lay in the range 14 to 18 cycles/sec. which is very near that given for harmine (15 to 20 cycles/sec.) by Hara and Kawamori (1954).

Antagonism of Harmine Tremor.—The results of Hara and Kawamori (1954) and Zetler (1957) indicate that harmine tremor can be effectively controlled by a variety of drugs, including anti-parkinsonian drugs, ganglion blocking agents, sedatives, ataraxics, anti-epileptics, 5-hydroxy-tryptamine and its antagonists. It is of interest to note that lysergic acid diethylamide, which resembles harmine in its chemical structure and which itself produces tremor, antagonizes the harmine tremor in very small doses (Zetler, 1957).

Lysergic Acid Diethylamide

Woolley (1956) has described the abnormal type of behaviour and tremor exhibited by mice after receiving lysergic acid diethylamide. After an initial moderate restlessness, the mice made backward movements with the hindlimbs outstretched and toes spread wide apart. The animals showed marked piloerection and changed their positions frequently on the floor of the cage, using their forelimbs only. They could not walk as could those treated with harmaline. After about 20 to 30 min., the mice remained quietly in a corner of the cage with hunched backs. Tremor appeared 5 to 10 min. after the intraperitoneal injection of lysergic acid diethylamide (4 mg./kg.) and it was fine, intermittent and interspersed with normal bodily movement (Fig. 3, lower record). The peak effect occurred between 8 and 15 min. after injection. The amplitude varied markedly, the frequency lying between 22 and 25 cycles/sec. The tremor index (Table I) indicates the severity of the tremor was much less than that following Tremorine or harmine.

Antagonism of Lysergic Acid Diethylamide.—Lysergic acid diethylamide tremor is not antagonized by 5-hydroxytryptamine or parasympathomimetic drugs (Woolley, 1956).

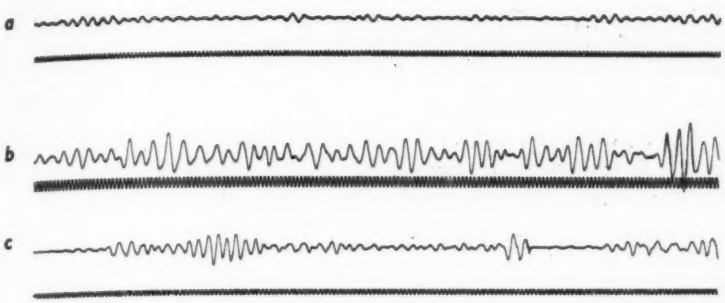

FIG. 3.—Cathode-ray oscillograph recording of (*a*) normal movement in the mouse, (*b*) tremor caused by 3-amino-1,1,3-triphenylpropan-1-ol (30 mg./kg.) and (*c*) lysergic acid diethylamide (4 mg./kg.). Time, 50 cycles/sec.

Aminotriphenylpropanol

Out of the twelve aminoalcohols synthesized in this laboratory, 3-amino-1,1,3-triphenylpropan-1-ol appeared to be the most potent tremor producing drug. The pharmacology of this aminoalcohol has been reported by Ahmed, Marshall, and Shepherd (1958).

In doses of 20 to 40 mg./kg. given intraperitoneally to mice, the drug produced the following effects: body extension, restlessness, hurried respiration, Straub tail phenomenon, and difficulty in walking forward. Tremor appeared within 8 to 15 min., occurring first in the head and then extending to the body and tail. The animal assumed an extended posture with outstretched limbs and the abdomen touching the floor. When tremor occurred there were continuous struggling movements to go forward, continuous extensions of the neck, body and forelimbs, but the hindlimbs failed to propel the body forward. The animal could, however, alter the position of the forepart of its body with its forelimbs and could make a circle lying in the same position. The animal frequently walked backward. The characteristic pattern of tremor associated with profound restlessness, struggling, ataxia and backward walking and circling movements were not seen with other tremor-producing drugs so far studied. Furthermore, one of the remarkable features of this compound was the production of severe hyperexcitability, an effect not seen with other drugs which produced tremor.

Tremor was present both at rest and during movement and was accentuated by external stimuli. The peak of the tremor occurred between 20 and 40 min. after the injection of the drug. The tremor was coarse, severe, and continuous, and lasted from 30 min. to 3 hr. Fig. 3*b* shows a tracing of aminoalcohol tremor, being characteristically irregular, with marked variations in rhythm and amplitude, sometimes distorted by struggling movements. The tremor was unlike that following harmine, harmaline, and Tremorine. With the tremor after lysergic acid diethylamide, such large amplitudes were not seen. The frequency of the aminoalcohol tremor ranged from 14 to 16 cycles/sec.

DISCUSSION

Table I shows the pattern of tremor and Table II the changes in general behaviour induced in mice by different drugs which produce tremor. The onset, amplitude, frequency, duration of effect and incidence of side effects varied considerably between the drugs. The technique of recording used enabled one type of tremor to be distinguished from another in a way which was not possible by direct observation of the animal. Harmine and harmaline give rise to a sinusoidal type of tremor pattern which closely resembled that of Tremorine but was not accompanied by the central parasympathetic stimulation caused by Tremorine. The tremor due to the aminotriphenylpropanol was in marked contrast to that due to the previous three drugs since it was completely irregular in amplitude and frequency. The record in this instance resembled, in many ways, an

TABLE II

COMPARISON OF EFFECTS PRODUCED BY DIFFERENT DRUGS IN MICE

– indicates absent, + slight and ++ marked effect.

Effect	Drugs						
	Tremorine	Harmine	Harmaline	Lysergic Acid Diethylamide	3-Amino-1,1,3-triphenyl-propan-1-ol	Eserine	Nicotine
Parasympathetic stimulation	++	–	–	–	–	+	+
Piloerection	–	–	+	++	–	–	–
Straub tail	+	+	+	–	+	+	+
Running movements	–	–	–	–	–	+	++
Walking backwards	–	–	–	+	+	–	–
Hyperexcitability	–	–	–	+	++	–	–

exaggeration of the normal movement of the animal. The tremor records after lysergic acid diethylamide had a superficial resemblance to those after aminotriphenylpropanol, but the tremor was less severe and the tremor index lower.

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OUABAIN-INDUCED VENTRICULAR TACHYCARDIA AND ITS EFFECT ON THE PERFORMANCE AND METABOLISM OF THE DOG HEART

BY

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At constant pressure work there was an increase in the oxygen consumption of the dog heart-lung preparation after tachycardia due to auricular stimulation and a far greater increase in consumption after ouabain-induced ventricular tachycardia, as compared with control hearts beating at their own sinus rhythm. In neither condition was the increase in coronary flow greater than the spontaneous increase in the controls. It is suggested that an increase in oxygen demand, under certain circumstances, may be met primarily by an increased desaturation of coronary blood. "Therapeutic" doses of ouabain did not improve the mechanical efficiency of the preparation. "Toxic" doses of ouabain which gave rise to ventricular tachycardia did not decrease the phosphocreatine or labile nucleotide phosphorus content of the heart provided there was no hypoxia of the heart muscle.

In intact dogs and in heart-lung preparations digitalis glycosides produce arrhythmias which usually lead to ventricular tachycardia and fibrillation. This paper deals mainly with the effect of ventricular tachycardia induced by ouabain on the performance and metabolism of the heart of the dog. Two groups of experiments served as controls: in one the ventricular rate was regulated by electrical stimulation of the auricle at the rate observed in the presence of ouabain; in the other, untreated control hearts beat at their own sinus rhythm.

METHODS

Male and female dogs were used weighing about 12 kg. Coronary flow was measured by our own modification of the Rodbard technique (Fawaz and Tutunji, 1957). This allows perfusion of both lungs with blood, direct measurement of coronary flow and oxygenation of the total "venous" blood in the reservoir. Oxygen consumption was determined by the direct Fick method. The oxygen content of the arterial and venous samples was estimated by the Van Slyke manometric method. In these experiments the lungs were ventilated with oxygen, but no oxygen was bubbled through the venous reservoir. Ouabain (1 : 100,000) was infused at the rate of 0.5 ml./min. The auricle was stimulated with monophasic impulses of 2 msec. duration from a Grass electronic stimulator. Experiments with ouabain lasted about 1 hr. and were terminated during the period of ventricular tachycardia a few minutes before ventricular fibrillation was expected. In a few experiments, blood samples were taken for analysis at the usual time and ventricular fibrillation was permitted, but in such cases the phosphorus compounds were not analysed. An experiment employing electrical stimulation followed every experiment with ouabain and the auricular stimulation was adjusted to duplicate as far as possible the changes in ventricular rate observed in the preceding experiment with ouabain. The control hearts beat at their own rhythm for 1 hr. The mean systemic arterial pressure was kept at about 100 mm. Hg and the total left ventricular output ranged from 800 to 1,000 ml./min. Other experimental details have been reported previously (Fawaz and Tutunji, 1957).

RESULTS

The results are summarized in Table I. In these experiments, unlike the control experiments reported earlier (Fawaz and Tutunji, 1957), a special attempt was made to keep the systemic arterial pressure as well as the work performed by the heart constant.

Coronary Flow and Desaturation of Coronary Blood.—It is common knowledge that coronary flow in a heart-lung preparation increases with time. In our experiments little change was observed after 20 min., but after 1 hr. the coronary flow was more than doubled. This was true of all three groups of experiments. Increased heart rate due to auricular stimulation has been reported to increase

TABLE I

EFFECT OF TACHYCARDIA ON ISOLATED DOG HEART

The numeral (1) indicates at the start of the experiment, (2) after 20 min., and (3) at the end of the experiment.

	No. of Expts.	Mean Ventricular Rate/min.			Mean Change in Arterial Pressure (mm. Hg)		Coronary Flow Mean±S.E. (ml./min.)			% Change in Work Performed Mean±S.E.		% Change in Oxygen Consumption Mean±S.E.		Mean O_2 Content of Coronary Venous Blood (vol. %)		Phosphorus Compounds of Heart Muscle Mean±S.E. (% of Total-Acid-Soluble-P)		
		(1)	(2)	(3)	(2)	(3)	(1)	(2)	(3)	(2)	(3)	(2)	(3)	(1)	(3)	Phospho-creatine	Inorganic P	Labile Nucleotide P (2/3 of ATP)
Controls . .	7	130	129	126	−0·1	−0·4	74±21	86±17	190±18	+0·46±2·2	−6·2±2·5	−0·84±2·9	−11·1±4·4	6·4	12·9	23·7±1·1	16·3±0·9	29·5±0·9
Ventricular tachycardia induced by ouabain	14	126	125	198	+1·1	−4·6	62±7	77±15	168±20	+6·3±5·1	−1·2±4·0	−0·56±4·1	+76±13·0	7·0	9·2	21·3±2·0	19·6±2·0	29·0±0·8
Tachycardia due to auricular stimulation	14	122	125	202	−0·5	−3·6	55±3	79±7	183±16	+1·6±1·2	−0·54±3·4	−0·54±2·8	+23·3±8·5	6·1	11·8	25·7±1·5	13·1±1·4	29·2±0·3

coronary flow in the intact dog (Laurent, Bolene-Williams, Williams, and Katz, 1956; Berglund, Borst, Duff, and Schreiner, 1958). It is of interest that in our experiments increased heart rate due to auricular stimulation did not increase coronary flow above the " spontaneous " increase observed in the controls. If the same heart rate was induced with ouabain, the oxygen consumption of the heart was still further increased, but this was not accompanied by a correspondingly greater increase in coronary flow. Clearly, the increased oxygen demand was met by a greater degree of desaturation of coronary blood.

It is not possible to predict in each case whether an increased oxygen demand by the heart will be met by increased coronary flow with constant arteriovenous oxygen difference, or by an increase in the latter. Both are possible and we have previously shown that increased cardiac oxygen consumption due to dinitrophenol could reduce the oxygen content of coronary venous blood to as little as 2 vol.% (Fawaz, Hawa, and Tutunji, 1957). It is generally assumed that the first response to elevated cardiac oxygen demand is an increase in coronary flow and that, when this is insufficient, an increased desaturation of coronary blood ensues. In the experiments with ouabain reported here, the latter mechanism came into play, for, though during ventricular tachycardia oxygen consumption was increased by an average of 76% in comparison with the controls, there was no greater increase in coronary flow. It may perhaps be more appropriate therefore to suggest that in the denervated heart the choice between the two mechanisms depends on the extent to which the coronary blood is desaturated at the time of the increase in oxygen demand. If the coronary venous oxygen content is high, as in the control experiments reported here, the increased oxygen demand can be met by increased desaturation without an increase in coronary flow. Further, it is possible that the nature of the stimulus to increased oxygen consumption largely determines the choice.

Oxygen Consumption and Mechanical Efficiency.— The mechanical efficiency of the control hearts, with a total left ventricular output of 800 to 1,000 ml./min., ranged between 8 and 10%. Within the first hour at least, there was no increase in the oxygen consumption of the control hearts and thus no change in " efficiency " (Table I). Starling and Visscher (1927) observed some decrease in the latter after 1 hr. However, their technique as well as their preparations differed from ours in such respects as total output (that of our hearts was twice as great as theirs) and load on the right heart.

The increased oxygen consumption in tachycardia due to auricular stimulation agreed with the findings of Laurent *et al.* (1956) and Berglund *et al.* (1958) on intact animals. However, ventricular tachycardia induced by ouabain led to a far greater increase in oxygen consumption and a corresponding decrease in mechanical efficiency.

Our experiments also throw some light on the question whether therapeutic doses of ouabain increase the mechanical efficiency of the heart. Twenty min. after the ouabain infusion, there was no change in oxygen consumption and thus no change in efficiency. Although the rate of ouabain infusion was not optimal we were still within the " therapeutic " range at 20 min. (Farah and Maresh, 1948); no irregularities were observed till then. We have repeatedly infused ouabain at this rate into heart-lung preparations to demonstrate its therapeutic potency in failure induced by a barbiturate and have convinced ourselves that, at 20 min., the " therapeutic " action of ouabain was evident. Rein (1949) reported that a heart-lung-liver preparation showed a greater mechanical efficiency than the classical preparation and he suspected the presence of digitalis-like substances in the liver which increased the efficiency of the heart. He also stated that the addition of digitalis acted in a way that resembled the inclusion of a liver in a heart-lung preparation. The experimental situation used by Rein (1949) was quite different from ours, but it was clear that under our conditions ouabain did not improve efficiency.

Phosphorus Compounds.—There was no significant change in the phosphocreatine or " labile nucleotide " phosphorus content of the left ventricular musculature after auricular stimulation or during ventricular tachycardia induced by ouabain. This is understandable when we examine the oxygen content of coronary venous blood (Table I). We have previously shown that myocardial phosphocreatine is particularly sensitive to even short periods of hypoxia produced by ischaemia or hypoxaemia (Fawaz *et al.*, 1957): in fact, we consider that a normal level of phosphocreatine indicates that the tissue has been properly handled before fixation. Wollenberger (1951) reported a decrease in the phosphocreatine content of the isolated dog heart during ventricular tachycardia induced by digitalis. However, in his experiments neither coronary flow nor coronary venous oxygen content was measured and the systemic flow was about half the value reported here. In view of the increased oxygen consumption observed after toxic doses, we suspect that an element of hypoxia was responsible for the low values for phosphocreatine found by Wollenberger (1951). Further, we had previously shown that the " resting " phosphocreatine values reported by Wollenberger (1951) for the isolated heart and particularly for the intact heart were too low (Fawaz and Hawa, 1953). The low phosphocreatine values reported by the same author for hearts at the onset of ventricular fibrillation induced by digitalis are obviously due to hypoxia (Fawaz *et al.*, 1957).

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THE PHARMACOLOGY OF THE ETHANESULPHONATE ANION

BY

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A method of preparing analytically pure sodium ethanesulphonate, $C_2H_5SO_4Na.H_2O$, in quantity is described. Physiological saline solutions were prepared in which a proportion of the sodium chloride normally present was replaced by an equimolar concentration of sodium ethanesulphonate. The effects of partial or total replacement of sodium chloride were examined upon a series of isolated organs, and the guinea-pig ileum preparation was found to be particularly sensitive to the change. Evidence is presented that these low-chloride solutions depolarized the cell membrane of smooth muscle, and that this effect might have been due to an alteration of the chloride ion potential.

The present work arose from a study of ventricular fibrillation in the Langendorff preparation of the rabbit heart. Alterations of the calcium or potassium ion concentrations in the solution perfusing this preparation could readily be made, without affecting any other properties of the perfusate. However, chloride ion forms such a high proportion of the total solute that any significant alteration of its concentration might change the tonicity, the *p*H, or the ionic strength of the solution.

Kärki (1958) replaced sodium chloride with a mixture of sodium sulphate and sucrose when he studied the effects of lowering the chloride ion concentration; however, this procedure might have altered the ionic strength of the solution, since the monovalent chloride ion was replaced by the divalent sulphate ion. We decided to examine sodium ethanesulphonate in order to find out whether it was pharmacologically inert: if it were, it might be used instead of sodium chloride to prepare low-chloride solutions. The ethanesulphonate was preferred to the methanesulphonate because the first member of a homologous series often has anomalous properties.

METHODS

Langendorff Rabbit Heart.—Rabbit hearts were perfused at 37° with the solution used by McEwen (1956) in a conventional Langendorff apparatus, and similar perfusion solutions were prepared in which a quarter, a half, or all the sodium chloride was replaced by an equimolar concentration of sodium ethanesulphonate. It was observed that calcium was precipitated from such a solution in which all the sodium chloride had been replaced when the solution was left standing for many hours. The solutions containing ethanesulphonate were therefore prepared from stock immediately before they were required; one stock solution contained all the solutes except calcium, and the second contained 20 g. of anhydrous calcium chloride dissolved in water to give 100 ml. of solution.

Rabbit Atria.—Atria were dissected carefully from freshly excised rabbit hearts, and set up at 29° in an isolated organ bath of conventional design (vol. 40 ml.). The original solution was that used by McEwen (1956), and changes were made in which a quarter, half, or all the sodium chloride was replaced by sodium ethanesulphonate.

Rat Ventricle Strip.—A strip of muscle 8 mm. long and 3 mm. broad was cut from the right ventricle of a freshly excised rat heart and suspended in Locke solution in an isolated organ bath (vol. 40 ml.). The muscle strip was cut from the pulmonary artery towards the apex of the ventricle parallel to the junction with the left ventricle; it was held flat in the bath by two spring clips, so that it presented the greatest area for oxygenation. The preparation was mounted to work a straw lever. Since it was not spontaneously active at 29°, it was stimulated through two electrodes placed parallel to the strip, one on each side of it; when a momentary current was passed the muscle gave an all-or-none response.

Rabbit Aorta.—A piece of rabbit aorta was cut in a spiral and unwound to form a long narrow strip of muscle which was set up in an isolated organ bath (vol. 40 ml.). The preparation had a rather slow response to drugs, and was gently stretched for two hours in normal Locke solution to develop tone. Submaximal doses of noradrenaline at 30 min. intervals produced slow contractions; this stimulation was repeated when all the sodium chloride in the solution was replaced by sodium ethanesulphonate.

Frog Rectus.—The conventional preparation was set up in a mixture of 5 vol. Locke solution with 2 vol. of water, and submaximal responses to acetylcholine were obtained. The sodium chloride in the Locke solution was then entirely replaced by sodium ethanesulphonate, and the same dose of acetylcholine was reapplied.

Guinea-pig Ileum.—The preparation was set up in conventional manner at 34° in Locke solution, and two test solutions were examined. The first of these was prepared with ethanesulphonate instead of chloride as in the other experiments which have been described; the second had normal sodium chloride, but ten times the normal concentration of potassium chloride.

Guinea-pig Taenia Coli.—Our colleague, Dr. G. Burnstock, was making an examination of the taenia coli muscle at this time, using the sucrose gap technique. He was kind enough to replace the sodium chloride in his normal perfusion solution with sodium ethanesulphonate whilst he recorded the membrane potential, the action potentials and the tension of the muscle.

Surface Tension and Densities of Solutions.—It was important to know whether the surface tension of Locke solution was altered when it was prepared from sodium ethanesulphonate instead of sodium chloride. A glass capillary tube was freed from grease in hot sodium dichromate solution, and washed through ten times with distilled water and four times with double-distilled water before drying at 110°. This tube, when cool, was dipped into the solution to be tested, which rose up to form a meniscus. The liquid level was then adjusted so that the meniscus came to a fixed point on the glass (in case the bore of the tube was not constant) and the capillary rise was recorded.

The densities of the solutions were determined by weighing in a density bottle.

Preparation of Sodium Ethanesulphonate

Sodium ethanesulphonate was required in substantial amounts, because of its high molecular weight relative to sodium chloride. The following procedure gave excellent yields of the pure salt and could be adapted to large scale preparation of the compound. A hot solution of the commercial barium salt (106 g. $(C_2H_5SO_3)_2Ba.H_2O$ in 1 l. H_2O) was treated with a slight excess of a hot solution of sodium sulphate (42 g. Na_2SO_4 in 400 ml. H_2O) and the precipitated barium sulphate was removed by filtration. The filtrate, which should give no precipitate with sodium sulphate solution, was concentrated in an open dish on a steam-bath until a thick crust of crystals formed on the surface of the hot solution. The cooled product formed an almost solid but damp mass of crystals, which was extracted with boiling methanol (500 ml.). The hot extract was filtered, cooled to 0° and the crystals collected. The insoluble material from the first extraction was re-extracted with the methanolic mother liquor from the first crop of crystals, the extract filtered and concentrated on the steam-bath until crystals appeared in the hot solution. On cooling to 0°, this gave a second crop of crystals. The total crude product so obtained (about 80 g.) was dissolved in boiling methanol (600 ml. +2 ml. H_2O) and filtered; it was concentrated until crystals appeared in the hot solution, and then cooled to 0°. This product was crystallized again in exactly the same manner; about 60 to 65 g. (70 to 76% yield) of sodium ethanesulphonate, free from barium and sulphate, were obtained. The salt was dried at atmospheric pressure over calcium chloride in a desiccator. (Found: C, 16.2; H, 4.4; H_2O, 10.0. Calculated for $C_2H_5SO_3Na.H_2O$: C, 16.0; H, 4.7; H_2O, 12.0%.) The method of recrystallization described was used because the salt crystallized so readily from its saturated solution in hot methanol that it was difficult to filter the solution. The salt could also be crystallized from a more dilute solution in methanol by the addition of acetone, but this method gave products with a variable content of water of crystallization. The anhydrous salt was hygroscopic and, as the analysis shows, it was difficult to dehydrate the hydrated salt completely at 100° *in vacuo*.

Results

A Locke solution, in which half the sodium chloride was replaced by sodium ethanesulphonate, will be referred to as "Locke ($\frac{1}{2}$E)"; similarly if all the sodium chloride in McEwen solution was replaced the solution will be referred to as "McEwen (E)."

Langendorff Rabbit Heart.—McEwen ($\frac{1}{2}$E) solution produced no marked change in the performance of this preparation, but when

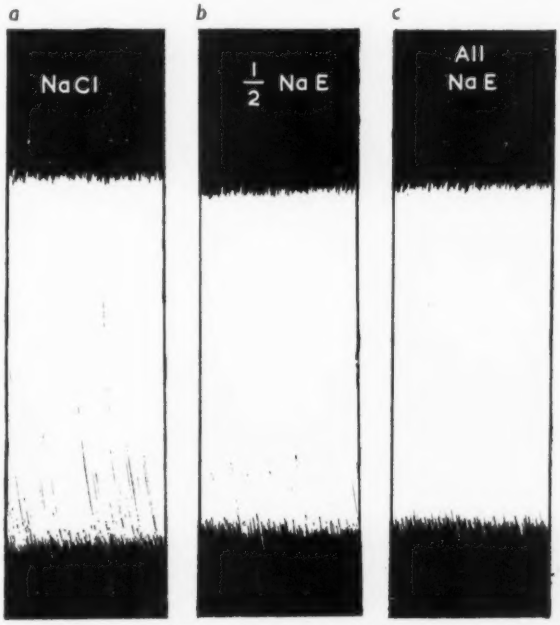

FIG. 1.—Isolated rabbit atria beating spontaneously at 29° in: (*a*) normal McEwen solution (**NaCl**); (*b*) McEwen solution in which half the sodium chloride was replaced by sodium ethanesulphonate (**½NaE**); (*c*) McEwen solution in which all the sodium chloride was replaced by sodium ethanesulphonate (**All NaE**).

McEwen (½E) solution was perfused there was always a fall in the coronary flow. The amplitude of the contractions sometimes diminished at this stage of an experiment, and the heart failed quickly in McEwen (E) solution; none of these effects could be readily reversed by changing back to normal McEwen solution.

We decided that the experiments which we had originally planned would not be possible, because the heart died in McEwen (E) solution. At the same time we thought it worth while to study the effect of replacing chloride by ethanesulphonate upon a number of other isolated organs.

Rabbit Atria.—Very little alteration was observed in the amplitude of contraction of rabbit atria when the solution was changed from normal McEwen to McEwen (½E) (Fig. 1), although in McEwen (E) solution there was an increased spontaneous rate of beating. Atria left overnight in the latter were still contracting the following morning at a quarter of their original amplitude.

Rat Ventricle Strip.—This preparation was driven at 40/min. for 0.5 min. periods, and then allowed 1.5 min. to recover (Fig. 2*a*). In normal Locke solution there was no spontaneous activity during the undriven periods (Fig. 2*a*), but activity was observed as soon as the bath was washed out with Locke (E) solution (Fig. 2*b*). This spontaneous rhythm continued even if all electrical stimuli were withdrawn (Fig. 2*c*), but could be abolished by the addition of acetylcholine to the bath.

Rabbit Aorta.—Isolated rabbit atria and rat ventricle behaved normally when all the sodium chloride in the solution was replaced by sodium ethanesulphonate. We therefore considered that the Langendorff heart preparation might have died because the coronary flow failed and not because ethanesulphonate had any direct toxic action upon the muscle.

Fig. 3 shows the contractions produced by submaximal doses of noradrenaline upon the rabbit aorta strip. When a change was made to Locke (E) solution the contraction due to noradrenaline was potentiated; however, after this contraction the muscle did not relax again to its original length. The aorta remained still more contracted after a second dose of noradrenaline in the modified solution, but relaxed again as soon as the bath was washed out with normal Locke.

Frog Rectus.—Submaximal acetylcholine contractures of the frog rectus were potentiated when

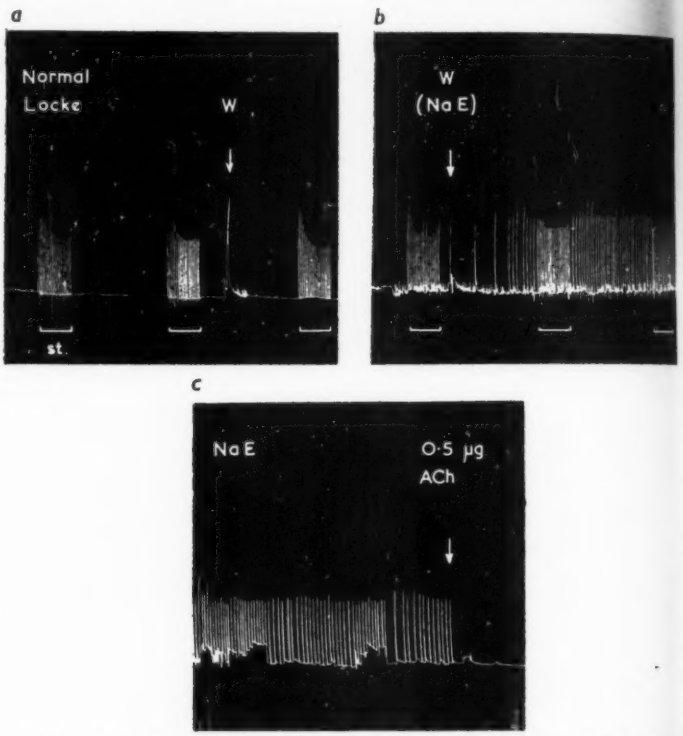

FIG. 2.—Isolated rat ventricle strip at 29°. (*a*) Driven for 30 sec. (for duration of horizontal bar) in Locke solution with 90 sec. rest periods: at W the bath was washed out with fresh Locke solution. (*b*) Driven as in (*a*). At W(NaE) the bath was washed out, and the normal Locke changed to a solution in which all the sodium chloride had been replaced by sodium ethanesulphonate. (*c*) No stimulation: the spontaneous activity in ethanesulphonate Locke was arrested by acetylcholine (ACh).

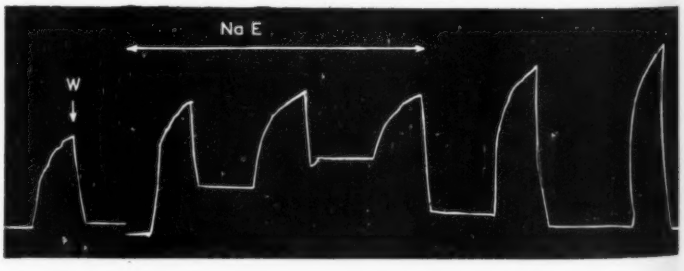

FIG. 3.—Rabbit aorta strip. Contractions in Locke solution after submaximal doses (0.3 µg.) of noradrenaline. When sodium ethanesulphonate Locke solution (NaE) was used the contractions were potentiated but the muscles did not relax fully.

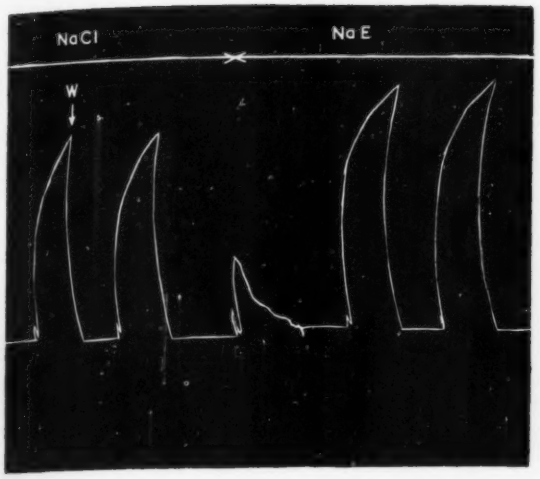

FIG. 4.—Frog rectus. Contractures after submaximal doses of acetylcholine (0.15 μg.). When the sodium chloride (**NaCl**) in the solution was replaced by sodium ethanesulphonate (**NaE**) there was a small contraction, and the response to acetylcholine was then potentiated. Bath volume, 5 ml.

the sodium chloride in the Ringer was replaced by sodium ethanesulphonate. The muscle contracted a little at the moment when the solutions were changed (Fig. 4), although this contracture was not maintained.

Guinea-pig Ileum.—A similar, but more powerful, contraction was observed when the Locke solution surrounding a piece of guinea-pig ileum was changed to Locke (E) solution. Again the contraction was not maintained, although it lasted much longer in this preparation than in the frog rectus.

Fig. 5*a* shows submaximal contractions of guinea-pig ileum produced by acetylcholine. When the muscle was fully contracted the kymograph was switched off and the bath was washed out with normal Locke solution (Fig. 5*a*, W). However, after the third contraction the bath was washed out with Locke (E), and the muscle did not relax but contracted still more. The kymograph was turned on at once, and the muscle was observed to relax slowly. When acetylcholine was applied in exactly the same way as before it had no effect.

This action of Locke (E) solution was entirely reversible, and Fig. 5*b* shows three acetylcholine contractions produced in normal Locke solution 1 hr. after the tracing in Fig. 5*a*. After the third contraction the bath was washed out with normal Locke solution ; when the muscle had relaxed the bath fluid was changed to one containing 10 times the normal potassium concentration. The muscle contracted, and then relaxed in a very similar fashion to the tracing in Fig. 5*a* ; once again acetylcholine was without effect.

Guinea-pig Taenia Coli. — The taenia coli preparation was composed of smooth muscle cells, and in this respect it was similar to the guinea-pig ileum. Depolarization of the muscle was observed when the modified Ringer solution was introduced during a sucrose-gap experiment ; action potentials were then discharged, tension was developed and was maintained for several minutes if the muscle was left in the ethanesulphonate Ringer. During this time added acetylcholine caused no additional depolarization, no increase in rate of firing of action potentials nor any increase in tension.

Surface Tension of the Solutions.—The surface tensions of Locke solution and Locke (E) solution were observed to be the same. However, when the two solutions were successively used in the same experiment, the gas bubbles from the oxygen tube were larger in Locke (E) than in normal Locke solution. This might have been due to a difference of surface tension which was too small to be measurable by the method described.

DISCUSSION

The first preparation studied was the rabbit heart, and although this died as soon as it was perfused with an ethanesulphonate solution isolated atrial or ventricular muscle appeared to function normally in the same solution. We concluded that the modified saline did not have any direct toxic action upon cardiac muscle and that the perfused hearts might have died from some other cause ; possibly this was the fall in coronary flow which had been observed when as little as half the sodium chloride had been replaced. We therefore decided to study the behaviour of arterial muscle, and observed that a strip of rabbit aorta would not relax fully when it was placed in a solution prepared with ethanesulphonate. This would explain the diminished coronary flow observed in perfused hearts, if the coronary vessels had behaved in a similar fashion.

The frog rectus preparation responded almost normally in ethanesulphonate solution, but the guinea-pig ileum was very sensitive to the change. Further, the effect on the ileum was very similar to that of an increased potassium concentration in the bath fluid. Since a high potassium concentration almost certainly acted by depolarizing the cell membrane, we thought that the sodium ethanesulphonate solution might act in a similar fashion, and we are very grateful to Dr. G. Burnstock, who tested this idea.

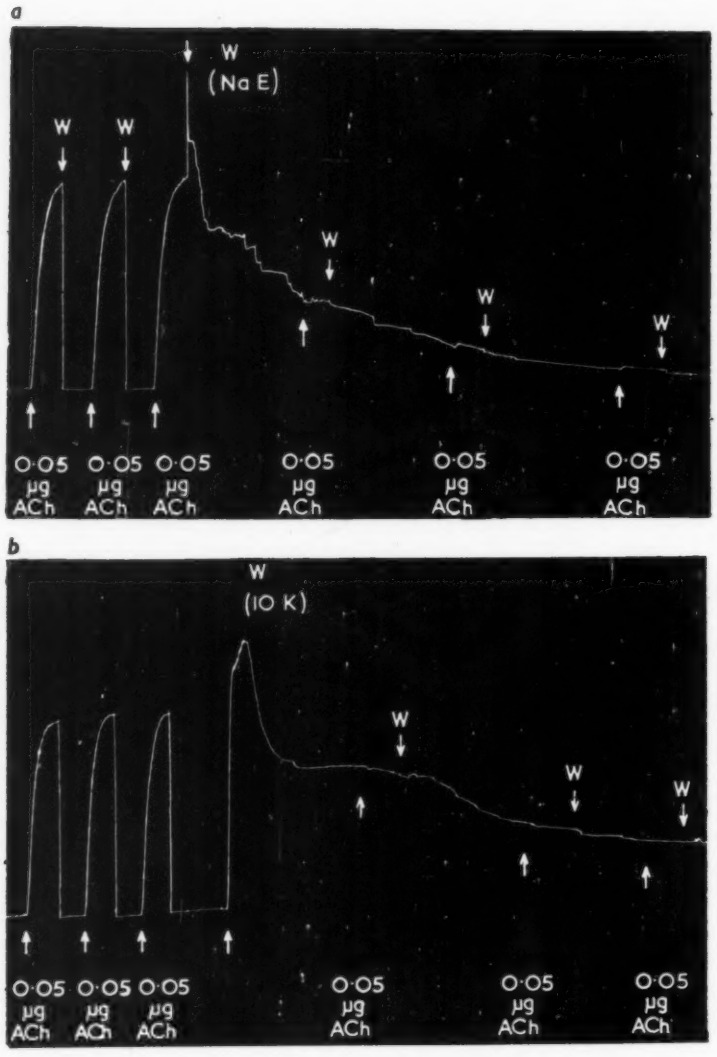

FIG. 5.—Guinea-pig ileum. Contractions after submaximal doses of acetylcholine: (*a*) after the third dose of acetylcholine the bath was washed out with Locke solution (E) prepared with sodium ethanesulphonate; (*b*) 1 hr. after Locke solution (E) had been replaced by normal Locke solution, acetylcholine produced its usual effect: after the third dose of acetylcholine the bath was washed out with normal Locke solution, and when the muscle had relaxed the fluid was changed to one containing ten times the normal potassium concentration (10K). Bath volume, 25 ml.

Dr. Burnstock observed a depolarization as soon as the sodium chloride in the solution superfusing the taenia coli was replaced by sodium ethanesulphonate; this led to the discharge of action potentials, and the muscle tension increased. Acetylcholine added at this stage did not cause further depolarization and did not increase the rate of firing of action potentials; it had no effect on the tension of the muscle, which in all respects behaved like the guinea-pig ileum preparation.

We therefore know that ethanesulphonate solution depolarized the cell membrane of smooth muscle; a similar action would explain all our other observations. Thus acetylcholine produced a contracture of the frog rectus preparation by depolarization, and this effect would be potentiated in ethanesulphonate solution if it partially depolarized the muscle. A short weak contracture of the rectus, when the solutions were changed, would also follow a partial depolarization; the noradrenaline contractions of the rabbit aorta would also be potentiated in the manner observed if noradrenaline acted by depolarization. Finally, a small depolarization of atrial muscle would reduce the membrane potential towards the threshold, and this action on the pacemaker would increase the spontaneous rate; a similar effect on the ventricle strip preparation would cause spontaneous activity. Both effects were observed.

However, in cardiac muscle the observations suggested that depolarization was only partial: that is to say, the membrane potential approached the threshold but did not pass it. Similarly in the frog rectus depolarization could only be partial or a much more powerful contracture would have been observed when the change was made to ethanesulphonate solution. On the other hand there was a much more complete depolarization in smooth muscle, so that many action potentials were fired and a powerful contraction took place. Why should there be this difference between smooth and other types of muscle?

In 1943 Goldman discussed the movement of ions in an ideal cell membrane, and Hodgkin and Katz (1949) were able to derive a very useful equation founded on his treatment. They showed that the electrical potential E across the membrane would be equal to

$$E=\frac{RT}{F}\ln\left[\frac{P_{K}(K)_{i}+P_{Na}(Na)_{i}+P_{Cl}(Cl)_{o}}{P_{K}(K)_{o}+P_{Na}(Na)_{o}+P_{Cl}(Cl)_{i}}\right] \quad \ldots\ldots (1)$$

where R=gas constant;
T=absolute temperature;
F=Faraday constant;
$(K)_i$, $(Na)_i$, and $(Cl)_i$ are ionic activities inside the cell;
$(K)_o$, $(Na)_o$, and $(Cl)_o$ are ionic activities outside the cell;
P_K, P_{Na}, and P_{Cl} are permeability constants for the ions in the cell membrane.

This equation involved a number of simplifying assumptions: E was the potential difference when there was no net ionic current through the membrane and when the ionic concentrations on each side of the membrane were uniform; that is to say, when there was no concentration gradient in the immediate neighbourhood of the membrane itself. Furthermore the ions in this ideal membrane were assumed to move under the influence of electrical and diffusion forces only.

However, all the results of the present work may be interpreted on the basis of this treatment simply as the result of changes in $(Cl)_o$. When a change was made from normal to ethanesulphonate solution, the chloride ion concentration outside the cell was reduced from about 140 mM. to 10 mM.; the activity of the chloride $(Cl)_o$ was similarly affected. Reduction of $(Cl)_o$ would reduce E according to equation (I), by reducing the value of the expression

$$P_K(K)_i+P_{Na}(Na)_i+P_{Cl}(Cl)_o \quad \ldots\ldots\ldots \text{(II)}$$

However, the significance of a change in $(Cl)_o$ would be determined by the relative values of the three permeability constants P_K, P_{Na}, and P_{Cl}.

In a resting membrane P_{Na} was much the smallest of these constants, and expression (II) approximated to

$$P_K(K)_i+P_{Cl}(Cl)_o \quad \ldots\ldots\ldots\ldots\ldots\ldots \text{(III)}$$

In smooth muscle P_{Cl} and P_K are thought to be comparable (Burnstock and Straub, 1958; Holman, 1958) so that changes in $(Cl)_o$ would alter E significantly; this was the situation which was observed in the taenia coli preparation, where the ethanesulphonate solution produced a profound depolarization. Moreover, should P_{Cl} be smaller than P_K in cardiac and skeletal muscle a smaller change in E would be expected from the same change of $(Cl)_o$; this is a likely interpretation of the observations on these tissues.

It is a pity that more evidence is not available concerning the ratio, $P_K : P_{Cl}$, in different cell membranes, as this information could confirm or confound the present suggestion. It is also possible that the action of ethanesulphonate was not primarily to alter $(Cl)_o$, but that the values of P_K, P_{Na}, and P_{Cl} were changed in the unusual environment. Should the ethanesulphonate solution diminish P_K and P_{Cl} selectively (so that P_{Na} assumed greater significance) E would become smaller, and this was the effect observed. A greater or lesser effect upon P_K and P_{Cl} in different tissues could again explain the observed differences between skeletal, cardiac, and smooth muscle.

This possibility could be tested by examining a short series of homologous sodium alkylsulphonates. In the series $C_nH_{2n+1}SO_3Na$ the physical properties should change in a regular manner as the value of n is increased; for example, solubility should decrease and surface active properties increase progressively. However, the latter should not become prominent for members with n less than 8, and micelle formation would not be expected to become apparent until n exceeded 12. Consequently, if a short series (say, n=2 to 6) were tested on isolated preparations as we have tested the ethane member, and no progressive change in the physiological effects was observed, it could be concluded that short-chain alkylsulphonate ions were pharmacologically inert and that the effects which we have observed were due simply to a change in the value of $(Cl)_o$. Alternatively, if the physiological effects changed progressively as the series was ascended, it would be clear that they depended not only upon the change in $(Cl)_o$ but also upon some specific action of the alkylsulphonate anion, such as an effect on the permeability properties of the cell membrane of the muscles, or an entry into the cell.

Long-chain alkylsulphonates would be unsuitable for replacing chloride because of their well-known surface active properties, but if short-chain alkylsulphonates could be shown to have no other effect than that due to a reduction in $(Cl)_o$ they might be used to replace chloride in the physiological solutions for some isolated preparations, though not for all as our results with the Langendorff rabbit heart demonstrated.

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OBSERVATIONS ON THE SUBSTRATE SPECIFICITY OF AMINE OXIDASES

BY

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A number of compounds have been tested as substrates of, first, the amine oxidases of rabbit and guinea-pig liver and of goat, pig, horse, and dog plasma and, second, the " diamine oxidase " of pig kidney. Of the three xylylenediamines [di(aminomethyl)benzenes] tested, *m*-xylylenediamine was found to be a substrate of the liver oxidase. All the plasma oxidases tested acted on both *m*- and *o*-xylylenediamine. Both 3- and 4-picolylamine (3- and 4-aminomethylpyridine) were readily oxidized by the liver enzymes; all the plasma oxidases tested oxidized 2-, 3-, and 4-picolylamine. 4-Picolylamine was more rapidly oxidized by horse and dog plasma than any other substrate hitherto examined.

Recent studies on the amine oxidases of mammalian plasma (Blaschko and Hawes, 1959; Blaschko, Friedman, Hawes, and Nilsson, 1959) have shown that the amine oxidases in general are a class of enzymes with a great diversity of substrate specificity. The inhibitor specificities of the plasma oxidases show that they are more closely related to the classical histaminase of pig kidney, although their substrate specificity is in many ways reminiscent of that of the intracellular amine oxidase.

In the following, experiments are reported on some new substrates of some of these oxidases. These substances were tested in the hope that they may be useful in the detection and characterization of these enzymes.

The enzymes tested in the present work were: the intracellular amine oxidase of mammalian liver, the histaminase (" diamine oxidase ") of pig kidney, the spermine oxidase of ruminant plasma (Hirsch, 1953; Tabor, Tabor, and Rosenthal, 1954), and the " benzylamine oxidase " of non-ruminant plasma (Bergeret, Blaschko, and Hawes, 1957).

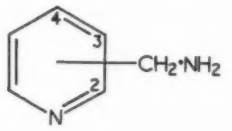
MATERIAL AND METHODS

Enzyme Preparations.—Dialysed extracts of guinea-pig and rabbit liver were prepared as described by Barlow, Blaschko, Himms, and Trendelenburg (1955). In a few preliminary experiments, carried out with Mrs. Jean M. Hagen (née Himms) in 1950, an acetone dried powder of rabbit liver was used as source of enzyme; this preparation, as well as the extract of pig kidney for the experiments on histaminase, has been described elsewhere (Blaschko and Hawkins, 1950). Sodium phosphate buffer of *p*H 7.4 was used throughout.

All the serum (or plasma) samples used, from the goat, pig, horse, or dog, were dialysed against 0.06 M-sodium phosphate buffer of *p*H 7.4. Serum was used in most experiments, but in a few instances we obtained fresh heparinized dog plasma from Professor I. de Burgh Daly and Dr. B. A. Waaler; the samples of dog serum were from animals used as blood donors in this department.

Substrates.—All compounds were used as the hydrochlorides. We are grateful to Dr. H. R. Ing, who in 1950 prepared for us the para and ortho isomers of xylylenediamine [di(aminomethyl)benzene, $C_6H_4(CH_2.NH_2)_2$]. The meta isomer has recently become available commercially from Messrs. Aldrich. We have also tested homo-*o*-xylylenediamine [*o*-(2-aminoethyl)benzylamine, $NH_2.CH_2.C_6H_4.CH_2.CH_2.NH_2$], kindly made for us by Dr. E. W. Gill, according to Helfer (1923).

The three isomeric picolylamines (2-, 3-, and 4-aminomethylpyridine) (I) were also obtained from Messrs. Aldrich.



(I)

Manometric Experiments—The rate of the enzymatic reaction was studied by measuring the initial uptake of oxygen after the addition of substrate at 37.5° in an atmosphere of O_2. Substrate concentrations after tipping were 10^{-2} M. In the experiments with liver amine oxidase, oxedrine [(±)-*p*-Sympatol] was used as the reference amine; with pig kidney enzyme, cadaverine; with goat plasma enzyme, spermine; with non-ruminant plasma enzymes (pig, horse, dog), benzylamine.

Results

Xylylenediamines

Liver Oxidases.—Of the three isomers of xylylenediamine, only the meta isomer was oxidized at a significant rate. Its oxidation was rapid in rabbit liver, but relatively slow in guinea-pig liver. The results of these experiments are summarized in Table I in which it can be seen that homo-*o*-xylylenediamine was oxidized more rapidly than *o*-xylylenediamine itself; with the latter compound, the rate of oxidation was scarcely significant. However, *o*-xylylenediamine was not without affinity for the liver enzyme: it strongly depressed the rate of oxidation of oxedrine. With *p*-xylylenediamine in equimolecular amounts, the oxidation of oxedrine by rabbit liver was only about halved.

TABLE I

OXIDATION OF XYLYLENEDIAMINES BY RABBIT AND GUINEA-PIG LIVER

Substrate concentrations, 10^{-2} M. The oxygen uptakes are given in % of the uptake with oxedrine as substrate, calculated from the first 30 min. of incubation. The number of separate experiments contributing to the mean is given in parentheses.

Substrate:	*p*-Xylylenediamine	*m*-Xylylenediamine	*o*-Xylylenediamine	Homo-*o*-xylylenediamine
Source of enzyme:				
Rabbit liver ..	0 (1)	63 (2)	9 (1)	14 (3)
Guinea-pig liver	5 (1)	21 (3)	3 (2)	17 (3)

Pig Kidney Oxidase ("*Diamine Oxidase*").—No oxygen uptake occurred when the pig kidney preparation was incubated with either *p*- or *m*-xylylenediamine; with *o*-xylylenediamine there was a small uptake of oxygen. The oxygen uptake in the presence of both cadaverine and *o*-xylylenediamine was practically the same as in the presence of cadaverine alone, but when cadaverine and either *p*- or *m*-xylylenediamine were present in equal concentrations (10^{-2} M), oxygen uptakes were completely abolished. The action of these two isomers on the oxidation of cadaverine was therefore also tested in lower concentrations, and the oxygen uptakes in the first 30 min. of incubation are given in Table II.

TABLE II

INHIBITION OF DIAMINE OXIDASE OF PIG KIDNEY BY *m*- AND BY *p*-XYLYLENEDIAMINE

The oxygen uptakes in the first 30 min. of incubation are given in μl. O_2. Cadaverine concentration, 10^{-2} M.

Xylylenediamine Isomer Tested	Cadaverine Alone	With 10^{-3} M Diamine	With 10^{-4} M Diamine
Meta	52	2·5	16
Para	68·5	7·5	43

In an experiment with homo-*o*-xylylenediamine, there was no significant uptake of oxygen; in the presence of both cadaverine and homo-*o*-xylylenediamine (10^{-2} M), the rate of oxygen uptake was about one-half that with cadaverine alone.

Oxidases of Non-Ruminant Plasma (Pig, Horse, and Dog).—No significant oxidation of *p*-xylylenediamine was seen, but both the meta and the ortho isomers were readily oxidized. Homo-*o*-xylylenediamine was not oxidized.

Although *p*-xylylenediamine was not oxidized by the three non-ruminant sera, it was not without affinity for the enzyme: the experiment of Table III shows that the oxidation of benzylamine by pig serum was inhibited by *p*-xylylenediamine.

TABLE III

INHIBITION OF THE PLASMA OXIDASES BY *p*-XYLYLENEDIAMINE

Oxygen uptakes in the first 30 min. of incubation are given in μl. O_2.

Source of Enzyme	Substrate	Substrate Alone (μl.)	Substrate with *p*-Xylylenediamine		
			10^{-2} M	10^{-3} M	10^{-4} M
Pig serum	10^{-2} M-benzylamine	48	0	24·5	43
Goat ,,	10^{-2} M-spermine	117	0	20·5	93

Ruminant Plasma Oxidase (Goat).—As with the non-ruminant sera, there was no oxidation of *p*-xylylenediamine, although the oxidation of spermine was inhibited by this isomer (see Table III). Both the meta and the ortho isomers were oxidized by goat serum at similar rates, about 1/3 of the rate with spermine as substrate. Homo-*o*-xylylenediamine was not oxidized.

Picolylamines

Liver Oxidases.—The rate of oxidation of the three picolylamines depended upon the position of the side chain: 2-picolylamine was scarcely oxidized, but both 3- and 4-picolylamine were oxidized rather rapidly. With rabbit liver extract the rate of oxidation of 3-picolylamine, the isomer more rapidly oxidized, was more than 1/2 of the rate with oxedrine; with guinea-pig liver, the rate was less, about 1/5 of that of oxedrine.

Pig Kidney Diamine Oxidase.—None of the three compounds was oxidized significantly.

Non-Ruminant Plasma Oxidases.—Sera of all the three species tested oxidized all the three picolylamines. Pig serum acted on 3-picolylamine most readily. With horse and dog sera the rates of oxidation increased in the order: 2-, 3-, 4-picolylamine. This is shown in Table IV, where oxygen uptakes within the first 30 min. of incubation with the three sera have been listed.

TABLE IV

OXIDASES OF NON-RUMINANT PLASMA

Rates of oxidation of the three isomers of picolylamine, in comparison with the oxidation of benzylamine. The values are μl. O_2 consumed in the first 30 min. Substrate concentration, 10^{-2} M.

Species	Benzylamine	2-Picolyl-amine	3-Picolyl-amine	4-Picolyl-amine
Pig	47·5	21·5	30·5	24·5
Horse ..	12·5	12·5	19·5	28
Dog ..	32	20·5	53·5	63

In both horse and dog sera the rates of oxidation of 3- and 4-picolylamine exceeded the rates of oxidation of benzylamine, hitherto considered the most readily oxidized substrate of the non-ruminant plasma oxidase. This observation has been used to confirm the finding (Blaschko *et al.*, 1959) that in different samples of dog serum (or plasma) the enzymatic activity is very different. Table V is from 14 experiments, in each of which a different

TABLE V

OXIDATION OF BENZYLAMINE AND 4-PICOLYLAMINE BY DIFFERENT SAMPLES OF DOG SERUM OR PLASMA

Substrate concentrations: 10^{-3} M. The values are μl. of O_2 taken up between the 5 and 30 min. readings. S indicates serum, P heparinized plasma, M male, F female.

Sex	Sample	Benzylamine (Bz)	4-Picolylamine (Pic)	Ratio $\frac{Pic}{Bz}$
?	S	37	69	1·9
M	P	6	18·5	3·1
M	P	5·5	27	4·9
F	S	4	16·5	4·1
M	S	9·5	22·5	2·4
?	S	30·5	51	1·7
F	P	17	32·5	1·9
M	S	9	27	3·0
M	S	11·5	16	1·4
M	S	12·5	33·5	2·7
M	S	4	16·5	4·1
M	S	28·5	84·5	3·0
M	P	5·5	22·5	4·1
M	P	7	19·5	2·8

sample of dog serum (or plasma) has been tested with both benzylamine and 4-picolylamine. It can be seen that the value of the ratio, rate of oxidation of 4-picolylamine/rate of oxidation of benzylamine, varied between 1.4 and 4.9. It must be borne in mind that the rate of oxidation of benzylamine by dog serum was very small and therefore subject to a relatively large experimental error.

Ruminant Plasma Oxidase.—Goat serum readily oxidized the three isomers of picolylamine. In one experiment, the oxygen uptakes in the first 30 min. of incubation were, with 10^{-2} M concentrations of spermine, 126 μl. O_2; of 2-picolylamine, 48 μl. O_2; of 3-picolylamine, 48 μl. O_2; of 4-picolylamine, 58 μl. O_2. These rates are similar in order to those found with the sera from non-ruminants.

DISCUSSION

These results confirm earlier work from this laboratory which showed that the amine oxidase of non-ruminant plasma is an enzyme distinct from the intracellular amine oxidase, an important catalyst in the inactivation of amines with biological activity, in particular the catechol amines and 5-hydroxytryptamine. The substrate specificities of the plasma enzymes, benzylamine oxidase and spermine oxidase, are known to be closely similar (Blaschko and Hawes, 1959; Blaschko *et al.*, 1959), a relationship also brought out in the present study. The liver enzymes acted only on the meta isomer of xylylenediamine, whereas the two plasma enzymes oxidized both the meta and the ortho isomers. Also with the picolylamines, there were more similarities between the plasma enzymes than between them and the liver enzyme. The xylylenediamines must now be added to the group of diamines that are more readily attacked by amine oxidases than by " diamine " oxidases.

As the outcome of this work, benzylamine is no longer the amine most readily oxidized by the plasma oxidases from non-ruminants. In both dog and horse serum, 4-picolylamine was found to be oxidized at a much faster rate than benzylamine. This has proved a useful observation: in the dog and the horse, plasma oxidase activity is relatively low, and the use of a substrate more rapidly oxidized than benzylamine makes the study of the enzyme in these species easier and it may help in the detection of enzyme in species in which it has not hitherto been found.

In the dog, the varying amounts of the plasma oxidase remain puzzling. Since it has been shown that the dog enzyme acts on histamine (Blaschko *et al.*, 1959), the possibility has to be considered that the enzyme is identical with the histaminase studied by Carlsten, Kahlson, and Wicksell (1949). These Swedish authors found little histaminase activity in the blood plasma, but rather larger amounts of enzyme in the thoracic lymph. Histamine is a rather poor substrate of the plasma oxidase in comparison with benzylamine and 4-picolylamine. Since dog lymph has not yet been tested for enzymatic activity with substrates other than histamine, it cannot at present be decided whether the enzyme in the dog lymph is identical with the plasma enzyme here studied. Carlsten (1950) has shown that the enzymatic activity in the thoracic duct is dependent upon adrenocortical activity: adrenalectomy leads to a considerable increase in enzymatic activity in the thoracic lymph. It is tempting to consider the large differences between samples of dog serum

or plasma as a consequence of differing levels of adrenocortical activity. This could be settled by a study of the enzyme from the thoracic duct itself or by studying the effect of adrenalectomy on the plasma enzyme. However that may be, the high rate of oxidation of 4-picolylamine, when compared with that of histamine or benzylamine, may serve as a reminder that the natural substrate of the plasma oxidase in non-ruminants is still unknown.

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THE EFFECTS OF LOADING RATS WITH INTRAVENOUS ISOTONIC SODIUM CHLORIDE ON THE RENAL RESPONSE TO DIURETICS

BY

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The effect of a number of diuretic agents was investigated on rats with different degrees of expansion of extracellular fluid volume. With moderate saline loading aminoisometradine and chlorthiazide were the most efficient diuretics. However, with a more severe saline load aminoisometradine and mersalyl had the greatest effect while chlorthiazide had no significant diuretic activity. The action of acetazolamide seemed independent of the saline load. Potassium chloride showed no diuretic activity. Injecting the drugs in pairs resulted in probable potentiation only with acetazolamide plus chlorthiazide. Since all the diuretics were used in doses which gave maximal effects, the summation of the effects of the other pairs of diuretics suggested different sites of action.

This investigation was to see whether certain diuretics acted as efficiently in animals loaded with saline as in normal animals. Saline loading expands the extracellular fluid space and, since diuretics are commonly used on subjects whose extracellular fluid compartment is expanded by the pathological retention of salt and water, these experiments should be of interest.

METHODS

All experiments were performed on unanaesthetized adult albino rats weighing between 175 g. and 210 g. Solutions of 5% dextrose or of 0.9% NaCl were infused continuously at a rate of either 0.075 or 0.75 ml./min. through cannulae inserted into the external jugular vein. This technique and the analytical procedures used have been described previously by Keeler and Schnieden (1958).

During the infusion at 0.075 ml./min. (slow infusion) urine was collected for 15 to 20 min. starting after about 100 min. from the beginning of the infusion. During the infusion at 0.75 ml./min. (fast infusion) urine was collected for 10 min. starting after 20 min.

Diuretic Agents.—Acetazolamide, aminoisometradine, and chlorthiazide were injected intravenously 20 min. before commencing the collection of urine. These drugs were given as 2.5% solutions in doses of 5 mg./100 g. body weight. Mersalyl was injected intravenously as a 0.5% solution (1 mg./100 g. body weight) 1 hr. before starting the collection period. Potassium was usually infused continuously as a 20 m.equiv./l. solution of KCl, but in some experiments KCl was injected as a 150 m.equiv./l. solution (75 μ.equiv./100 g. body weight) 2 min. before starting the collection period.

The fluid load retained by the animals was estimated by noting the change in weight during the infusion.

RESULTS

Table I shows the effects of various diuretics on the excretion of sodium, potassium, and water by normal rats during an infusion of 5% dextrose solution at 0.075 ml./min. or of 0.9% NaCl solution at a rate of either 0.075 or 0.75 ml./min. Whereas the infusion of 5% dextrose solution was not accompanied by retention of fluid (as indicated by mean % body weight change, Δ wt.), the infusion of 0.9% saline led to considerable fluid retention. The output of sodium by control animals receiving fast infusions of saline was nearly 40 times greater than the output of sodium by control animals receiving 5% dextrose solution. The excretion of potassium was 5 times greater and the inulin clearance (glomerular filtration rate) 1.9 times greater.

The Effect of Diuretics

Potassium.—The infusion of 20 m.equiv./l. KCl under the three conditions of fluid loading resulted in changes in sodium output ($U_{Na}V$) and urine flow (V) which were not statistically significant. The expected rise in potassium output (U_KV) was present (Table I, Fig. 1).

In view of this result, the effect of an acute loading with 75 μ.equiv. KCl/100 g. body weight was investigated during a fast saline infusion. Although the plasma potassium level was suddenly raised from a mean of 4.85 to 7.5 m.equiv./l., there was no statistically significant change in the excretion of either sodium or water, sodium output being 22.9 μ.equiv./min. and urine flow 159 μl./min.

Acetazolamide.—Fig. 1 and Table I show that the injection of acetazolamide during the infusion

TABLE I

THE EFFECTS OF DIURETICS UNDER DIFFERENT CONDITIONS OF FLUID LOADING

The table shows effects on urine flow (V, μl./min.), sodium and potassium excretion ($U_{Na}V$, U_KV, μ.equiv./min. respectively), inulin clearance (G.F.R., ml./min.) and mean % body weight change (Δ wt.). All results are expressed in terms of 100 g. body weight, and are mean values $\pm$ standard error.

	(*a*) 5% Dextrose (0·075 ml./min.) Δ wt. $= -0{\cdot}31 \pm 0{\cdot}15$					(*b*) Saline (0·075 ml./min.) Δ wt. $= 2{\cdot}1 \pm 0{\cdot}53$					(*c*) Saline (0·75 ml./min.) Δ wt. $= 7{\cdot}3 \pm 0{\cdot}41$				
	No. of Rats	V	$U_{Na}V$	U_KV	GFR	No. of Rats	V	$U_{Na}V$	U_KV	GFR	No. of Rats	V	$U_{Na}V$	U_KV	GFR
Controls ..	16	45·0 ± 2·6	0·60 ± 0·07	0·36 ± 0·05	0·92 ± 0·03	16	29·5 ± 2·3	4·44 ± 0·30	0·70 ± 0·045	1·00 ± 0·050	8	182 ± 13·5	22·8 ± 1·2	1·82 ± 0·04	1·65 ± 0·10
KCl	16	40·6 ± 1·6	0·57 ± 0·07	0·96 ± 0·03	0·96 ± 0·04	9	27·3 ± 1·5	4·45 ± 0·31	1·26 ± 0·086	0·97 ± 0·025	8	171 ± 19·3	21·8 ± 1·3	5·34 ± 0·3	1·58 ± 0·05
Acetazolamide	14	62·3 ± 3·8	4·30 ± 0·45	2·21 ± 0·07	0·71 ± 0·02	13	62·7 ± 2·7	9·25 ± 0·30	1·74 ± 0·104	0·82 ± 0·04	9	232 ± 11·7	26·3 ± 0·8	2·50 ± 0·10	1·21 ± 0·07
Aminoisometradine	8	68·5 ± 5·8	4·39 ± 0·51	1·10 ± 0·08	0·96 ± 0·10	8	81·5 ± 6·1	12·14 ± 0·76	1·21 ± 0·103	0·78 ± 0·051	8	272 ± 14·3	30·1 ± 1·4	2·08 ± 0·26	1·55 ± 0·09
Mersalyl ..	8	38·9 ± 4·5	1·84 ± 0·26	0·72 ± 0·08	0·76 ± 0·03	8	30·3 ± 2·7	5·21 ± 0·68	0·84 ± 0·085	0·81 ± 0·036	8	265 ± 17·6	28·7 ± 1·9	1·58 ± 0·17	1·51 ± 0·04
Chlorthiazide ..	8	87·0 ± 6·7	4·91 ± 0·52	1·81 ± 0·12	0·87 ± 0·03	8	77·5 ± 3·4	11·4 ± 0·63	1·46 ± 0·065	1·10 ± 0·062	8	165 ± 5·04	25·8 ± 1·6	2·07 ± 0·10	1·51 ± 0·07

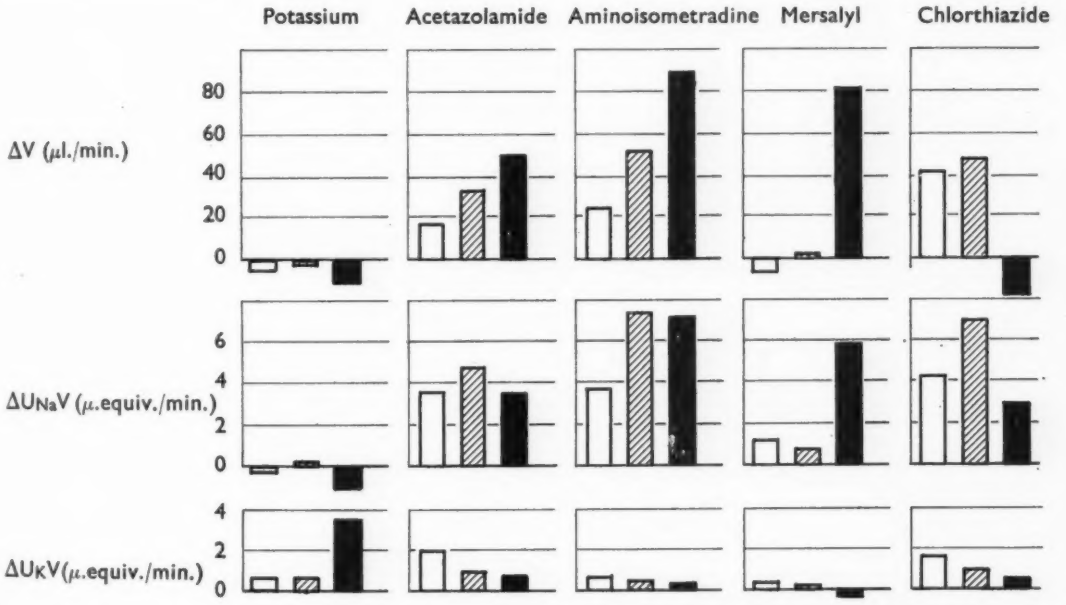

FIG. 1.—The effects of some diuretics on urine volume (ΔV) and sodium and potassium excretion ($\Delta U_{Na}V$ and ΔU_KV respectively) under the three conditions of fluid loading. Results shown as diuretic output minus control output. Mean values have been taken from the groups shown in Table I. Blank columns, 5% dextrose; cross-hatched columns, saline (0.075 ml./min.); and solid columns, saline (0.75 ml./min.).

of 5% dextrose solution increased the mean sodium output by 3.7 μ.equiv./min. above control values. Similar increments were noted after acetazolamide during both slow and fast saline infusions. The size of the increase was apparently independent of the control value. The size of the rise in urine flow following acetazolamide was independent of the control levels but roughly proportional to the mean % body weight change (Δ wt.).

Aminoisometradine.—In animals receiving 5% dextrose solution, aminoisometradine increased sodium output by 3.8 μ.equiv./min. However, when injected during the slow and fast saline infusions aminoisometradine increased the mean sodium output by 7.7 and 7.3 μ.equiv./min. respectively. The difference between the increments in sodium output caused by aminoisometradine during 5% dextrose solution and slow or fast saline infusions is significant ($P<0.05$). The change in urine flow was independent of control levels but proportional to Δ wt.

Mersalyl.—In these experiments mersalyl had little or no effect on urine flow or sodium output during the infusion of 5% dextrose solution or of 0.9% NaCl at 0.075 ml./min. During fast saline infusions, however, mersalyl increased urine flow by 83 μl./min. and sodium excretion by 5.9 μ.equiv./min.

Chlorthiazide.—Chlorthiazide given during the infusion of 5% dextrose solution increased the mean sodium output by 4.3 μ.equiv./min. and the mean urine flow by 42 μl./min. The corresponding values during slow saline infusions were 7.0 μ.equiv./min. and 48 μl./min.

These values were similar to those obtained after acetazolamide or aminoisometradine. However, when chlorthiazide was injected during fast saline infusions there was no significant change in either sodium or water output ($P>0.1$ in each instance).

Three observations were common to experiments with all the diuretics. Firstly, the control values (Table I) show that as the fluid load was increased so did the potassium output. Secondly, the increments in potassium excretion produced by acetazolamide, aminoisometradine, mersalyl, or chlorthiazide in every experiment decreased as the fluid load was raised (Fig. 1). Thirdly, the concentration of sodium in the urine ($U_{Na}V/V$, derived from Table I) of animals receiving 0.75 ml. of saline/min. was very much lower than that of animals receiving saline at only 0.075 ml./min., although the sodium concentration in the infusate was 154 m.equiv./l. in each experiment. For example, the mean urinary sodium concentration of control rats during slow saline infusions was 150 m.equiv./l.; during fast saline infusions it was 125 m.equiv./l.

Diuretic Combinations.—To get further information on the mechanism of action of the diuretics, they were injected in pairs during fast saline infusions. Each diuretic was used in the same dosage as in the previous experiments. The results of these combined injections on urine flow and on sodium and potassium output are shown in Table

TABLE II

(*a*) THE EFFECTS OF COMBINED DOSES OF DIURETICS ON THE EXCRETION OF SODIUM, POTASSIUM AND WATER (MEAN ± S.E.) DURING LOADING WITH SALINE AT 0.75 ML./MIN.					(*b*) THE DIFFERENCES BETWEEN THE EXCESS SODIUM EXCRETION ABOVE CONTROL VALUES FOR COMBINED DIURETICS ($\Delta U_{NaV(a+b)}$) AND THE SUMS OF THE EXCESS OUTPUTS FOR EACH DIURETIC SEPARATELY ($\Delta U_{NaV(a)} + \Delta U_{NaV(b)}$)	
Diuretic Combination	No. of Rats	V	$U_{Na}V$	$U_{K}V$	$\Delta U_{NaV\,(a+b)} - (\Delta U_{NaV(a)} + \Delta U_{NaV(b)})$	*P*
Chlorthiazide + Acetazolamide	6	201 ± 13·1	32·7 ± 2·45	2·10 ± 0·102	+3·4	<0·05
Chlorthiazide + Aminoisometradine	6	211 ± 16·2	35·2 ± 2·45	2·14 ± 0·128	+2·1	≃0·1
Chlorthiazide + Mersalyl	6	221 ± 17·5	35·8 ± 1·67	1·60 ± 0·092	+4·1	>0·05
Acetazolamide + Aminoisometradine	9	224 ± 22·4	30·4 ± 2·54	2·00 ± 0·083	−3·2	>0·1
Acetazolamide + Mersalyl	8	223 ± 14·0	30·8 ± 2·08	1·93 ± 0·129	−1·4	≃0·2
Aminoisometradine + Mersalyl	6	238 ± 21·9	33·7 ± 2·69	1·85 ± 0·157	−2·3	>0·1

II*a*, and should be compared with the results of the individual drugs given under the same conditions and shown in Table I*c*.

Preliminary experiments had shown that the doses of the drugs given individually produced maximal diuretic effects in rats with fast saline infusions as twice the dose produced no significant changes in diuresis; it seemed therefore of interest to calculate for sodium output whether the net increase above control levels for each combination of drugs was statistically different from the sum of the net increases in sodium output for each of the drugs given alone. The results are shown in Table II*b*. *P* values were obtained by analysis of variance. With the possible exception of acetazolamide given with chlorthiazide ($P<0.05$) no significant potentiation of action was observed. This suggests that these two drugs had different sites of action.

Discussion

The interpretation of the results involves two assumptions, that weight changes indicate the amount of retained fluid and that isotonic saline is distributed throughout the extracellular fluid compartment (taken as 20% of the body weight). If these assumptions are true, it will be seen from the mean % changes in body weight (Δ wt. in Table I) that rats receiving 5% dextrose solution had practically normal extracellular fluid volumes, whilst the mean expansion of the extracellular fluid compartment by slow infusions of saline was 10% and that by fast infusions was 36%. It is clear therefore that the diuretics are acting under three very different conditions.

Effects of Diuretics on Urine Flow and Sodium Excretion.—The effects of acetazolamide on sodium output seemed to be relatively independent both of the degree of fluid loading and of the sodium load presented to the tubules (Table I). Since Maren, Mayer, and Wadsworth (1954) have shown that acetazolamide is a powerful inhibitor of carbonic anhydrase, the present results suggest that the carbonic anhydrase mechanism in the kidney may be responsible for the reabsorption of a relatively constant amount of sodium.

Although the salts of potassium are frequently said to be diuretic, the infusion of as much as 7.5 μ.equiv. KCl/min./100 g. body weight failed to change significantly the rate of sodium and water output. Fuller, Macleod, and Pitts (1955) noted that potassium loading at a similar rate in dogs had relatively little effect on sodium output.

The most surprising observation arose from a comparison of the effects of mersalyl and chlorthiazide under the three conditions of fluid loading. During the slow infusion of 5% dextrose solution or of saline, mersalyl had practically no diuretic action whatever (Fig. 1). However, when the fluid load was raised by fast saline infusion, the diuretic activity of mersalyl became apparent. Pitts, Duggan, and Miner (1950) have argued that, if mersalyl acted on the distal tubules, then failure of mersalyl to cause diuresis may be related to a low glomerular filtration rate. It will be noted, however, that acetazolamide, which almost certainly acts upon the distal tubules (Pitts, 1958), caused a diuresis even though the fall in inulin clearance (glomerular filtration rate) was similar in size to that after mersalyl.

In contrast to mersalyl, the action of chlorthiazide was similar to that of aminoisometradine during slow dextrose solution or saline infusion. However, during the rapid infusion of saline chlorthiazide failed to change significantly either urine flow or sodium output.

It was pointed out in the results that the sodium concentration in the urine of rats infused with saline at 0.75 ml./min. was considerably less than its concentration in the infusate (154 m.equiv./l.). This accords with the theoretical predictions of Cole and Meredith (1957).

Effects of Diuretics on Urinary Potassium Excretion.—Koch, Brazeau, and Gilman (1956) have observed that, when sodium excretion was increased by isotonic saline infusion, the excretion of potassium was also raised. Similar results were noted in the present experiments when the saline load was increased in control animals (Table I).

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ISOLATED RABBIT ATRIA WITH SYMPATHETIC NERVE SUPPLY

BY

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The atria of the rabbit heart have been isolated together with their sympathetic nerves, so that the latter could be stimulated and the effect on the rate and amplitude of beating could be measured. Cocaine and phenoxybenzamine increased the response to stimulation. When the preparation was from rabbits previously given reserpine, stimulation of the sympathetic nerves caused inhibition. This inhibition was increased by eserine and abolished by atropine.

McEwen (1956) has described a preparation of rabbit atria with the vagus nerves attached. An attempt has now been made to set up rabbit atria with the sympathetic nerves and to study the action of cocaine, phenoxybenzamine, acetylcholine, and ganglionic blocking agents upon it. Some rabbits were given reserpine beforehand.

METHODS

The rabbit was killed by cutting off the head with strong shears as high up the neck as possible and was fixed on its back on the operating table. The skin covering the neck was cut along the midline. The carotid artery, vagus, and cervical sympathetic nerves and internal jugular vein were tied and cut at the cranial end as a single bundle, and liberated from the surrounding connective tissue. Holding the caudal end of the tied nerves, artery, and vein laterally, the blunt end of a scalpel was inserted between them and the trachea and oesophagus. This was done on both sides. Holding the bundles together in the midline, a cut was made with strong scissors through the dorsal part of the first rib and continued to the last rib on each side. Then the front of the thorax was removed leaving the thymus and adipose tissue attached to the heart. Lifting the heart upwards, the vertebral column was cut through transversely at the third thoracic vertebra and a second cut was made between the first and second thoracic vertebrae. The preparation was then freed quickly from skeletal muscle, and put into oxygenated Locke solution at 30°.

The preparation was left in the solution for 5 min., then taken out, freed from muscle, and fixed on an L-shaped Perspex rod by tying the remaining piece of vertebral column to the foot of the L-shaped rod. The preparation was returned to the Locke solution. With the preparation immersed and with continuing oxygenation the cervical sympathetic nerve was identified in the bundle containing the carotid artery and vagus nerve and a thread was attached to it so that it could later be passed through the electrode. One nerve wa cleaned with forceps as far as the stellate ganglio without cutting any branches of that nerve. (Th other nerve was kept in reserve.) The stellate ganglio was freed from surrounding tissue as far as possibl The trachea and oesophagus were removed; adipos tissue and the thymus were pulled away with the finger (If a preparation was required for stimulation of th vagus as well as the sympathetic nerve, the trachea an oesophagus were not removed.) The pericardium wa cut and the ventricles were cut away. The aorta an pulmonary artery were cut through close to the auricles A thread was passed through the apices of both auricles for attachment to a lever. The sympathetic nerve wa passed through electrodes similar to those for stimulatio of the phrenic nerve in the rat diaphragm preparatio (Bülbring, 1946). The nerve passed through two smal platinum rings 1 mm. apart embedded in a piece o Araldite cement on the end of a glass tube containin the insulated leads. In this tube there was also a 0.5 mm. polyethylene tube which terminated between the tw platinum rings. The other end of the polyethylene tub carried the electrodes out of the glass tube over th edge of the bath, and hung outside so that fluid fro the bath syphoned over at about 2 drops/min. thu keeping the nerve oxygenated.

The thread from the atria was attached to a sprin lever which carried a platinum wire on its under side. When the lever was pulled down by a contraction, th wire dipped into a small trough containing saline an completed a circuit through a post office counter drive by a 120 V dry battery. The atrial beats were the recorded on the counter and the number of beats/min was observed.

Stimulation was applied in the early experiments fo 2 min., but in the later ones mostly for 15 sec., usin pulses of 0.5 to 1.0 mA., and 1 to 2 msec. duration at frequency of 10/sec. Since hexamethonium (1.2 mg./ml did not reduce the atrial contractions, the stimulatio was postganglionic.

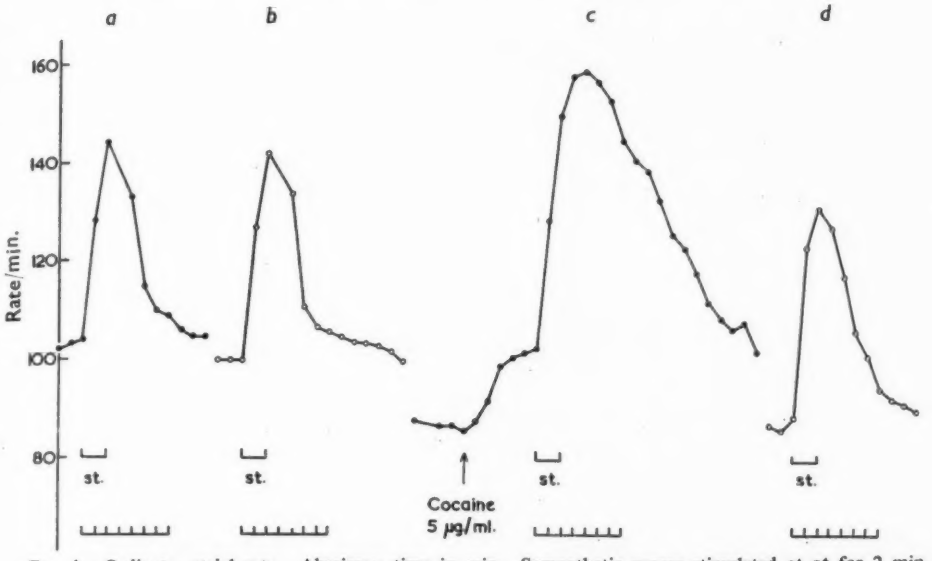

FIG. 1.—Ordinate, atrial rate. Abscissae, time in min. Sympathetic nerves stimulated at st for 2 min. causing rise in atrial rate. *a* and *b*, control observations. *c*, in presence of cocaine (5 µg./ml.). *d*, after washing out cocaine.

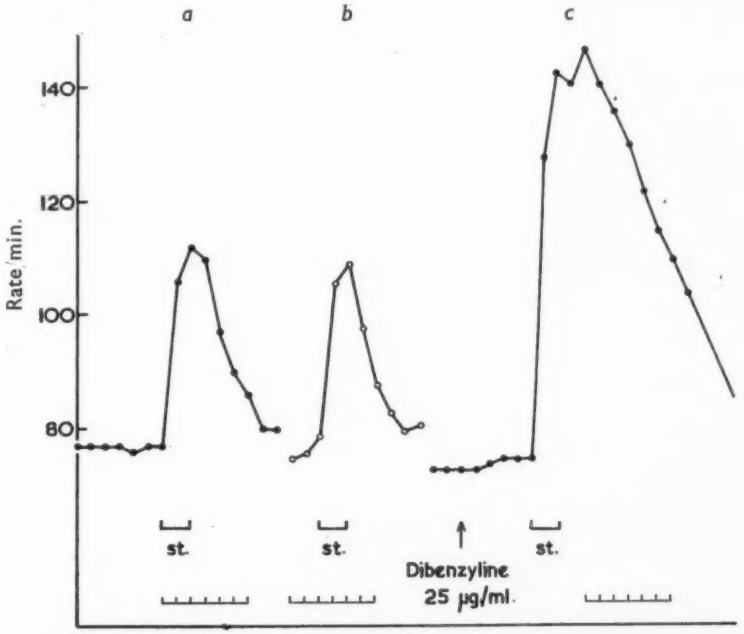

FIG. 2.—Co-ordinates as in Fig. 1. *a* and *b* show rise in atrial rate after sympathetic nerve stimulation for 2 min. *c* shows rise in presence of 25 µg./ml. of phenoxybenzamine (Dibenzyline).

RESULTS

Effect of Cocaine.—The effect of stimulating the postganglionic fibres on the rate of atrial contraction is illustrated in Fig. 1. Stimulation for 2 min. (Fig. 1*a* and *b*) caused an acceleration in which the rate rose from about 100 to 140/min., and returned in the next 2 to 3 min. The two effects were very similar.

In a series of preparations, cocaine (2×10^{-6} g./ml. and upwards) increased the effect of stimulation on the rate. Curve *c* (Fig. 1) shows the much greater effect of stimulation in the presence of 5×10^{-6} g./ml. of cocaine. The increase was almost twice as great as in the control and the duration was about 7 min. When cocaine was removed from the bath, the effect of

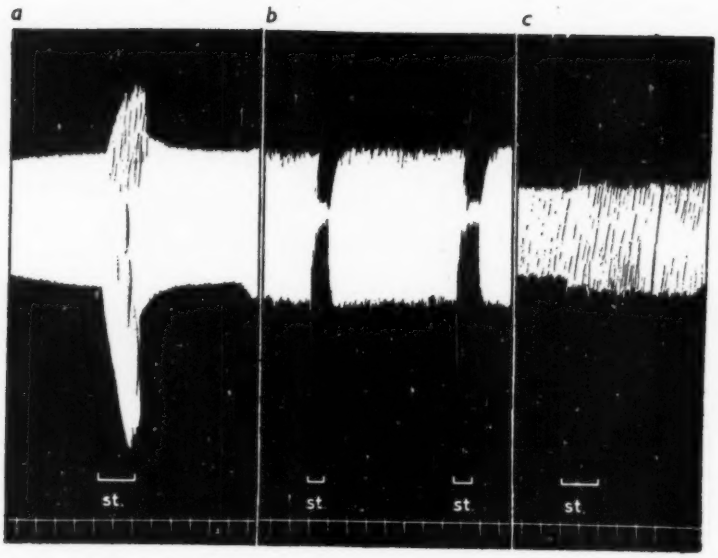

FIG. 3.—Kymograph record of atrial contractions. *a*, Effect of sympathetic nerve stimulation (st) for 2 min. on atria from normal rabbit; *b*, effect of sympathetic nerve stimulation for 1 min. on atria from rabbit treated with reserpine; *c*, effect of stimulation on the same preparation as in *b* after addition of atropine. Time, 1 min.

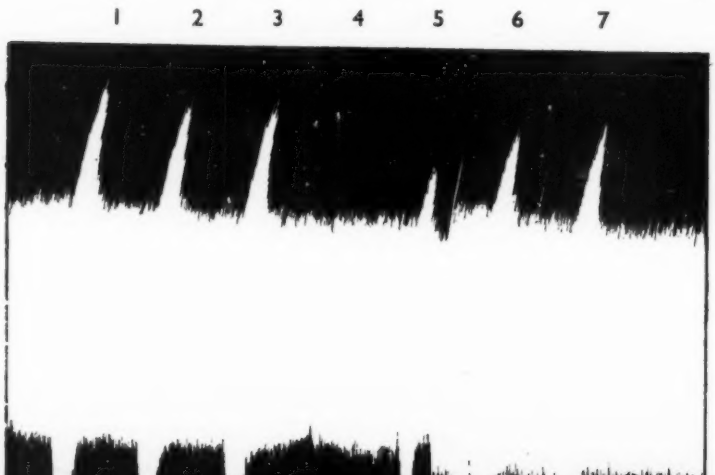

FIG. 4.—Kymograph record of atrial contractions showing the effect of sympathetic nerve stimulation for 15 sec. (at white rectangles) in the presence of atropine (30 µg./ml.). After the addition of acetylcholine (0.5 mg./ml.) to the bath stimulation 4 was without effect, and stimulation 5 had only a small effect. After this stimulation the bath was washed out (W), atropine being replaced. Time, 30 sec.

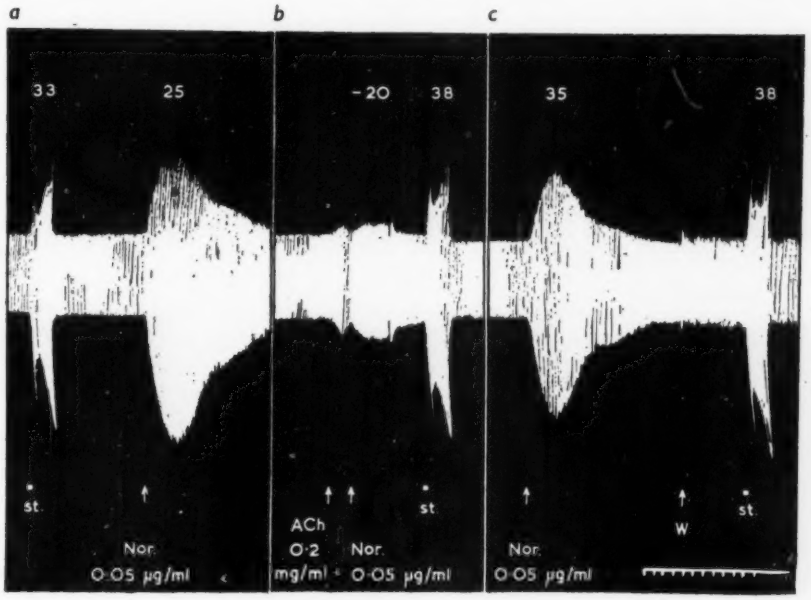

FIG. 5.—Effect of sympathetic nerve stimulation (st) and of noradrenaline (Nor) on the atrial contractions. The numerals at the top of the record show the change in atrial rate. All observations were made in the presence of atropine (30 μg./ml.). In *b* the addition of acetylcholine (0.2 mg./ml.) prevented the increase in rate by noradrenaline but not that due to sympathetic nerve stimulation. In the presence of acetylcholine noradrenaline caused a decrease in rate. Time, 30 sec.

stimulation returned to its initial dimensions (curve *d*).

Effect of Phenoxybenzamine.—Observations were also made with phenoxybenzamine (Dibenyline, Dibenzyline, N-phenoxyisopropyl-N-benzyl-β-chloroethylamine), which modified the effect of stimulation in the same way as cocaine. When 2.5×10^{-5} g./ml. of phenoxybenzamine was added to the bath, the increase in rate was much greater and its duration was longer. The effect persisted when the phenoxybenzamine was removed from the bath. An example of the action is given in Fig. 2.

Effect of Previous Administration of Reserpine.—Preparations of atria were made from rabbits treated for 2 days beforehand with reserpine, 1 mg./kg. on the first day and 2 mg./kg. on the second day intraperitoneally. In all preparations, the increase in rate due to sympathetic nerve stimulation was very small or absent, and in 5 out of 10 stimulation caused a slowing of the rate (Fig. 3). In *a* the effect of stimulation in a normal preparation is shown, in *b* the inhibition produced in a preparation from a reserpine-treated rabbit, and in *c* the absence of effect after adding atropine to the bath. The inhibitory action shown in Fig. 3 *b* was increased by eserine, and was clearly due to the liberation of acetylcholine.

Effect of Quinidine.—Quinidine, which has been shown by many workers to reduce the action of adrenaline on blood vessels, blocked the effect of sympathetic stimulation in a concentration of 20 to 30 μg./ml., the effect being restored when quinidine was removed from the bath.

Effect of Acetylcholine.—Some evidence has been published showing that postganglionic stimulation of sympathetic fibres can be blocked by ganglionic blocking agents (Brücke, 1935; Varagić, 1956) and observations were therefore made on atria. When hexamethonium was added to the bath, there was no diminution of the effect of stimulation even when the concentration was as high as 1.2 mg./ml. Pempidine also showed no evidence of block. With acetylcholine, however, some block was observed. When atropine was first added to the bath to exclude this inhibition, a high concentration of acetylcholine (0.2 to 0.5 mg./ml.), which itself had no effect on the heart rate, blocked the effect of sympathetic stimulation. This is illustrated in Fig. 4, which shows the increased amplitude of contraction caused by three successive stimulations of the postganglionic fibres for periods of 15 sec. in the presence of atropine, 30 μg./ml. When acetylcholine was added in the high concentration of 0.5 mg./ml., stimulation 1 min. later was without effect, and 3 min. later produced only a small effect. Acetylcholine was then removed from the bath, atropine being replaced, and stimulation then produced its original effect.

It was next observed that in the presence of the high concentration of acetylcholine the effect of noradrenaline in causing a rise in the atrial rate was blocked while stimulation was unaffected. This is illustrated in Fig. 5 for which the concentration of noradrenaline was 0.05 μg./ml. In the presence of atropine, acetylcholine (0.2 mg./ml.) blocked the action of noradrenaline (0.05 μg./ml.)

though it did not block the effect of stimulation. After removing the acetylcholine the effect of noradrenaline returned.

Discussion

Observations on tissues isolated together with their extrinsic nerve supply have increased in number in the last few years. Garry and Gillespie (1955) described a preparation of the rabbit colon with sympathetic and parasympathetic nerves, and in this department three different preparations have now been made. Varagić (1956) described a preparation of the rabbit uterus with the hypogastric nerves; McEwen (1956) described the setting up of the isolated heart and of the isolated atria with the vagus nerves, and in the present work the isolated atria has been used with the sympathetic nerves attached.

Certain similarities in the sympathetic innervation of these tissues may first be noted. The addition of cocaine to the bath greatly increased the effect of sympathetic stimulation both on the rabbit uterus and on the atria, and so did the addition of anti-adrenaline substances. Thus Varagić (1956) found that tolazoline increased the effect on the uterus of stimulating the hypogastric, and I found that phenoxybenzamine increased the effect of stimulating the sympathetic nerves to the atria. Recently Brown and Gillespie (1957) have shown that when the splenic nerves were stimulated at a frequency of 10/sec. the amount of noradrenaline liberated in the venous effluent was increased in the presence of phenoxybenzamine. It seems probable that the greater effect of stimulation on the uterus and on the atria was due to a similar increase in the amount of noradrenaline able to act on the uterine and atrial receptors.

The observations on the different tissues have been similar in another respect, namely that they have suggested the presence of cholinergic fibres in the sympathetic nerves. After Dale and Feldberg (1934) had shown that the fibres to the sweat glands were cholinergic, Bülbring and Burn (1935) gave evidence of cholinergic fibres to the muscles of the dog hindleg, and then Bacq and Frédericq (1935) stated that there were cholinergic fibres in the sympathetic supply to the cat nictitating membrane. Recently Varagić (1956) reported that, when he stimulated the hypogastric nerves to the uterus, the addition of eserine increased the size of the contractions and the addition of atropine reduced it. The effect of eserine was, however, seen in only two out of fourteen experiments. Gillespie and Mackenna (1959) have found that, when the isolated colon was prepared from a rabbit treated with reserpine, the inhibitory response to stimulation of the sympathetic nerves was converted to an excitatory response which was abolished by atropine. In the present experiments with atria from rabbits treated with reserpine, stimulation of the sympathetic nerve fibres has caused inhibition in half of them. This inhibition was increased by eserine and abolished by atropine. Thus the number of organs in which some of the sympathetic innervation appears to be cholinergic steadily increases. It thus appears that it is still too early for a final statement concerning postganglionic sympathetic nerve fibres. We do not yet understand the relation between those which are adrenergic and those which are cholinergic.

Finally, it was observed that sympathetic stimulation of the atria could be blocked by very high concentrations of acetylcholine, which, however, also blocked the stimulant action of noradrenaline. The experiments were done because Brücke (1935) found that the pilomotor response in the cat tail to stimulation of the lumbar sympathetic chain was blocked at the site where acetylcholine was injected into the skin, and because Varagić (1956) found that hexamethonium blocked the response of the rabbit uterus to hypogastric stimulation. Since in the atria acetylcholine not only blocked sympathetic stimulation but also the effect of noradrenaline, its action had no relation to block at a synapse.

This work has been done during the tenure of a British Council scholarship. The work was suggested by Professor J. H. Burn, and done under his supervision. I am very grateful to him.

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MECHANISM OF ACTION OF ORAL ANTI-DIABETIC DRUGS

BY

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The fall in blood sugar with carbutamide (BZ55) 24 hr. after the maintenance dose of insulin (28.1% ±2.9 s.e.) in rabbits made severely diabetic with alloxan was significantly different from the change in the blood sugar with the same drug given 72 hr. (6.8% ±2.0) or distilled water given 24 hr. (7.7% ±1.3) respectively after the last dose of insulin. It is postulated that the drug acts by liberating bound insulin in the plasma.

In spite of the widespread use of carbutamide, the mechanism of action of this drug is uncertain. Work done in this laboratory (Aiman and Kulkarni, 1957) showed that carbutamide potentiated the action of insulin on the rat diaphragm. This finding suggested that the drug might act on the binding of insulin with the plasma proteins. Sirek and Best (1956) have shown that the growth hormone of the anterior pituitary may release bound insulin, because it causes a fall in the blood sugar of depancreatized dogs if injected 24 hr. after, but not if injected 72 hr. after, the last maintenance dose of insulin.

This work was undertaken to demonstrate a similar effect of carbutamide on bound exogenous insulin given to severely diabetic rabbits.

METHODS

Six male rabbits weighing between 1 and 2 kg. were used. They received a standard diet fed for a fixed number of hours daily. The rabbits were rendered diabetic by intravenous injections of 180 mg./kg. of body weight of alloxan monohydrate after a fasting period of 48 hr. If the diabetes showed regression, alloxan injections were repeated till the fasting blood sugar was at least 300 mg./100 ml.

After diabetes had been established for eight days with a fasting blood sugar of about 300 mg./100 ml. or more, 1 unit of insulin zinc suspension (Lente) was given daily for a further eight days, during which the fasting blood sugar was maintained between 190 and 280 mg./100 ml.

Then the blood sugar values were studied over a period of 7 hr. hourly except the first and fourth hours in the following manner: (1) On the first day after 50 ml. of distilled water had been given orally; (2) on the fifth and seventh days after 500 mg./kg. of body weight of carbutamide [BZ55, Nadisan (Neo-Pharma Private Ltd., Bombay)] had been given orally, but having omitted the usual insulin injections on the fifth and sixth days. (3) On the third day, only the fasting blood sugar was determined. Insulin was then restarted and continued for 4 days and the blood sugar curves were studied as before, but giving 50 ml. of distilled water 72 hr. after the last dose of insulin.

The method of Folin and Malmros (1932) was used for blood sugar estimations.

RESULTS

The mean blood sugar values are given in Table I. The maximum % fall in blood sugar in each rabbit under the various experimental conditions are given in Table II, in which it can be seen that the difference between the falls after carbutamide and those after distilled water differ significantly ($P > 0.01$). Fig. 1 shows the mean fall in each of the experiments in which the fasting blood sugar was adjusted to equal 100 mg./100 ml.

TABLE I

MEAN BLOOD SUGAR VALUES

Mean blood sugar values ± s.e. of mean. The numerals in parentheses indicate the number of animals used.

Samples	Mean Blood Sugar Values			
	With Distilled Water		With Carbutamide	
	24 Hr. after Insulin (6)	72 Hr. after Insulin (3)	24 Hr. after Insulin (6)	72 Hr. after Insulin (6)
Fasting blood sugar ..	242 ± 13·2	277 ± 11·4	238·5 ± 14·6	294 ± 5·4
After 2 hr.	245 ± 15·3	290 ± 10·9	230·9 ± 16·4	299 ± 8·2
,, 3 ,,	233 ± 13·2	312·6 ± 23·9	206·5 ± 12·9	294·7 ± 10·5
,, 5 ,,	225 ± 12·6	284 ± 13·6	188·5 ± 13·3	285·9 ± 10·5
,, 6 ,,	231 ± 15·5	283 ± 9·1	179·7 ± 13·8	274·8 ± 3·2
,, 7 ,,	236 ± 14·8	299 ± 21·8	185·5 ± 13·9	282·0 ± 5·1

TABLE II

MAXIMUM PERCENTAGE FALL IN THE BLOOD SUGAR UNDER THE VARIOUS EXPERIMENTAL CONDITIONS

The maximum % fall in Col. 1 when compared with that in Col. 3 is significant ($t=6.1$; $P<0.01$). The maximum % fall in Col. 1 when compared with that in Col. 2 is significant ($t=6.2$; $P<0.01$). The maximum % fall in Col. 2 when compared with that in Col. 3 is not significant ($t=0.4$; $P>0.5$).

Rabbit No.	Maximum Percentage Fall in the Blood Sugar (mg./100 ml.)		
	With Carbutamide		With Distilled Water
	24 Hr. after Insulin	72 Hr. after Insulin	24 Hr. after Insulin
	1	2	3
1	38·8	7·9	10·1
2	25·0	9·8	8·6
3	33·2	9·7	7·4
4	22·8	10·0	9·3
5	18·0	7·7	8·5
6	30·6	4·5	2·3
Mean ± s.e. of mean ..	28·1±2·99	6·8±2·0	7·7±1·3

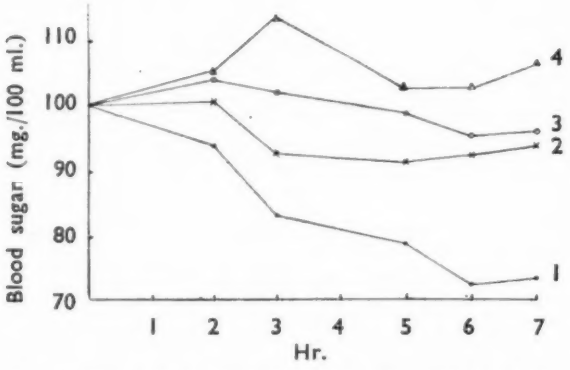

FIG. 1.—Mean falls in blood sugar with carbutamide and distilled water in diabetic rabbits 24 and 72 hr. after the last dose of insulin. The fasting blood sugar was adjusted to 100 mg./100 ml. **1**, with carbutamide 24 hr. after insulin. **2**, with distilled water 24 hr. after insulin. **3**, with carbutamide 72 hr. after insulin. **4**, with distilled water 72 hr. after insulin.

From these results it can be seen that carbutamide given 24 hr. after insulin produced a marked and significant fall in the blood sugar when compared to the fall with the same drug given 72 hr. after insulin.

DISCUSSION

The rabbits were used about three weeks after the alloxan injections when they were severely diabetic as can be seen from the fasting blood sugar estimations in Table I, and hence there was very little chance of endogenous insulin being present in any appreciable quantity; whatever effect was obtained with the drug was therefore due to an action other than through the β cells.

Carbutamide was used in the present study because the mechanisms of action of carbutamide and of tolbutamide (D860) are probably similar and because carbutamide can be administered more easily than can tolbutamide. The effect of the insulin used lasted up to 24 hr. and all experiments were therefore carried out at least 24 hr. after the last dose of insulin.

Recently Randle and Taylor (1958), Marsh *et al.* (1952) and Bornstein (1953) have shown that insulin can be bound to plasma proteins, but the factors determining the unbinding have yet to be clearly demonstrated. Welsh, Henley, Williams, and Cox (1956) have reported that insulin labelled with [^{131}I] disappeared less rapidly from the plasma of diabetic than from that of non-diabetic patients and the binding action of diabetic plasma with insulin reduced its hypoglycaemic action in mice.

In our experiment the fall with carbutamide given 24 hr. after insulin was much more marked than that with carbutamide given 72 hr. after insulin, or that with distilled water given 24 hr. after insulin (Fig. 1). Also the fall with carbutamide given 72 hr. after insulin was not significantly different from that with distilled water given 24 hr. after insulin (Table II). To substantiate our findings, the blood sugar curves were carried out with distilled water given 72 hr. after insulin in three rabbits (Table I). The change in blood sugar in this condition when compared with the change with carbutamide given 72 hr. after insulin showed no significant difference ($t=1.75$, $P>0.1$). Hence it would appear that, since the action of carbutamide was dependent upon the interval after the last dose of insulin, it acted by potentiating insulin in some way.

Our results suggested the possibility that carbutamide produced a significant fall in blood sugar when given 24 hr. after insulin, by influencing any insulin in bound form in a manner similar to that reported by Sirek and Best (1956) with growth hormone in depancreatized dogs. While it is also possible that the exogenous insulin "permitted" the hypoglycaemic effect of a direct action of carbutamide on the liver to reduce its glucose output (Ricketts, Wildberger, and Schmid, 1957), the results obtained in studies in which insulin was added to incubation flasks containing rat diaphragms (Aiman and Kulkarni, 1957) support a potentiating effect rather than a "permissive" liver action. The sulphonylureas are bound to plasma proteins. Is it possible that this binding in some way affects the equilibrium of bound insulin and that the direct pancreatic action (Loubatières, 1955; Pozza *et al.*,

1956; Ashworth *et al.*, 1956) may be an indirect effect resulting from altered plasma insulin levels?

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THE ACTION OF ANTISYMPATHOMIMETIC DRUGS ON THE URINARY EXCRETION OF ADRENALINE AND NORADRENALINE

BY

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(RECEIVED MAY 11, 1959)

In cats anaesthetized with pentobarbitone sodium, phenoxybenzamine, dibenamine, phentolamine, and piperoxane reduced the blood pressure and increased the urinary excretion of noradrenaline. When the fall of blood pressure after phenoxybenzamine was prevented by repeated injections of vasopressin, the urinary excretion of noradrenaline did not rise. Hydergine and hydrallazine reduced the blood pressure without raising the urinary excretion of noradrenaline. In dogs, the infusion of small amounts of noradrenaline led to a significantly higher urinary recovery of the amine after phenoxybenzamine than before. It is concluded that antisympathomimetic drugs interfere with the destruction in the body of noradrenaline, whether released reflexly in hypotension or injected.

It had been found previously that phenoxybenzamine raised the urinary excretion of adrenaline and of noradrenaline in dogs (Benfey, Ledoux, and Melville, 1958). Hexamethonium, which markedly reduced the accompanying hypotension, prevented this effect. A similar hypotension produced by repeated injections of methacholine only elevated urinary adrenaline moderately (Benfey, Ledoux, and Melville, 1959). Similarly, phenoxybenzamine raised the plasma adrenaline and noradrenaline concentration in dogs (Millar, Keener, and Benfey, 1959).

In the earlier studies, phenoxybenzamine lowered the blood pressure for a prolonged period of time, and evidence was obtained that hypotension in the absence of anti-adrenaline agents differed from that in the presence of antisympathomimetic substances in the urinary excretion of adrenaline and noradrenaline in dogs. Whereas in the former there was only a moderate rise of adrenaline, in the latter there was a markedly increased adrenaline and noradrenaline excretion. It appeared to be of interest to investigate the effects of antisympathomimetic agents in the absence of hypotension. As experiments with dogs were unsuccessful we used cats. In addition, previous experiments to demonstrate the action of phenoxybenzamine on the urinary recovery of infused noradrenaline in dogs have been modified. At the autumn meeting of the American Society for Pharmacology and Experimental Therapeutics in 1957 we reported that antisympathomimetic drugs increased the urinary recovery of injected adrenaline and noradrenaline (Benfey, Mazurkiewicz, and Melville, 1958a).

METHODS

Cats were first anaesthetized with ether. The femoral vein was cannulated as quickly as possible, and the anaesthesia was continued with intravenous pentobarbitone sodium. After opening the abdomen, the bladder was cannulated with polyethylene tubing. Dogs were anaesthetized with pentobarbitone sodium and, after opening the abdomen, the ureters were cannulated with polyethylene tubing. The blood pressure was recorded from the femoral artery and an infusion of 0.9% NaCl solution was given into a femoral vein at a constant rate (0.2 ml./kg./min.) throughout the experiment. Total urine samples were collected every 30 min. The urine was passed through a column of alumina and adrenaline and noradrenaline were determined by the fluorimetric method described by Millar and Benfey (1958). The clearance of *p*-aminohippuric acid was determined according to Smith, Finkelstein, Aliminosa, Crawford, and Graber (1945) and that of creatinine according to Folin and Wu (1919).

Drugs were given after a control period of 60 min. which permitted two 30 min. control collections. The following agents were employed: phenoxybenzamine hydrochloride (Dibenzyline, Smith, Kline, and French) was dissolved in a small quantity of 95% ethanol,

brought to a volume of 20 ml. with physiological saline and injected intravenously over a 10 min. period; vasopressin (Benfey, 1953); phentolamine (Regitine hydrochloride), and hydrallazine (Apresoline hydrochloride, Ciba); piperoxane and hexamethonium bromide (Vegolysen, Poulenc). The drugs were kindly supplied by the companies named, and the Sterling Winthrop Research Institute kindly gave us (−)-noradrenaline bitartrate monohydrate. The amounts of adrenaline and noradrenaline given below refer to the base; they are not corrected for such errors in the method of determination as losses on purification of the urine samples.

Results

In cats, the application of 10 mg./kg. of phenoxybenzamine led to an increase in the urinary excretion of noradrenaline, but the output of adrenaline was not influenced (Fig. 1). This is in contrast to previous observations on dogs which showed an equal rise of noradrenaline and adrenaline; however, the rise in urinary noradrenaline in cats was of the same order as that in dogs (Benfey *et al.*, 1959). The blood pressure dropped under the influence of the drug and remained at about 90 mm. Hg for at least 3 hr.

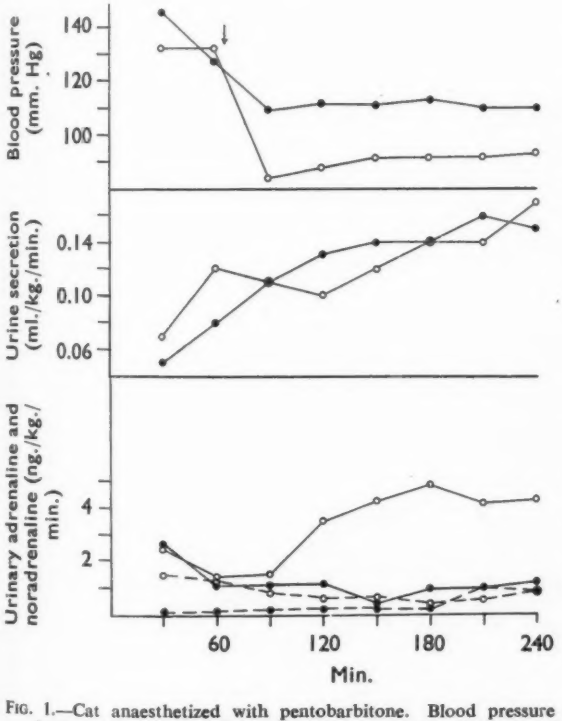

FIG. 1.—Cat anaesthetized with pentobarbitone. Blood pressure (top), urine flow (middle) and urinary excretion (bottom) of adrenaline (broken line) and noradrenaline (full line). At arrow, phenoxybenzamine (10 mg./kg.). O, without, and ●, with repeated injections of vasopressin (2 to 10 units). Mean of 5 experiments.

Intravenous injections of vasopressin (2 to 10 units at intervals of at least 10 min.) maintained a fairly stable blood pressure after phenoxybenzamine in five out of eight cats. In dogs this was achieved in only one out of six animals for a limited period of time. As seen in Fig. 1, maintenance of the blood pressure at approximately 110 mm. Hg prevented the increased urinary excretion of noradrenaline following phenoxybenzamine in cats. The total urinary excretion of noradrenaline in 3 hr. following injection of phenoxybenzamine was 667 ± 153 without, and 166 ± 83 ng./kg. ± S.E. with, vasopressin. The difference is statistically significant ($P < 0.05$). The rate of urine secretion was not affected by vasopressin.

Dibenamine similarly reduced the blood pressure to an average of approximately 80 mm. Hg and raised the urinary excretion of noradrenaline, but it also elevated moderately the urinary output of adrenaline (Table I).

TABLE I
URINARY EXCRETION OF ADRENALINE AND NORADRENALINE, RATE OF URINE SECRETION, AND BLOOD PRESSURE IN CATS BEFORE AND AFTER DIBENAMINE

Means of four experiments. 30 min. periods.

Period	Adrenaline (ng./kg./min.)	Noradrenaline (ng./kg./min.)	Urine (ml./kg./min.)	B.P. (mm. Hg)
Control 1 and 2	0·55	0·95	0·04	141→117
After dibenamine (20 mg./kg.) 3 to 10	2·5	4·7	0·07	70→85→67

Phentolamine (Table II) and piperoxane (Table III) increased both adrenaline and noradrenaline excretion as did dibenamine. However, the first two drugs reduced the blood pressure only moderately, to a mean of approximately 100 mm. Hg. In contrast, hydergine did not influence the urinary excretion of adrenaline and noradrenaline although it reduced the blood pressure (Table IV).

Finally, we tried to reduce the blood pressure by repeated intravenous injections of hydrallazine in amounts of 1 to 5 mg./kg. A mean of approximately 105 mm. Hg was obtained. Hydrallazine has little if any antisympathomimetic action. There was a moderate rise of both adrenaline and noradrenaline (Table V). The excretion of noradrenaline did not greatly exceed that of adrenaline.

The urine from the cat bladder obtained after opening the abdomen and before the bladder was cannulated was assayed. It contained a mean of 4.9 ng./ml. of adrenaline and 29.3 ng./ml. of noradrenaline in ten samples of a mean volume of 19 ml. In contrast, urine obtained from dogs

TABLE II

URINARY EXCRETION OF ADRENALINE AND NORADRENALINE, RATE OF URINE SECRETION, AND BLOOD PRESSURE IN CATS BEFORE, DURING, AND AFTER PHENTOLAMINE INFUSION

Means of four experiments. 30 min. periods. Phentolamine infusion, 2·5 mg./kg./30 min.

Period	Adrenaline (ng./kg./min.)	Noradrenaline (ng./kg./min.)	Urine (ml./kg./min.)	B.P. (mm. Hg)
1 and 2	1·4	1·9	0·09	140→131
Infusion started				
3 to 8	2·4	5·3	0·11	105→93→107
Infusion stopped				
9 and 10	3·0	4·6	0·15	98→95

TABLE III

URINARY EXCRETION OF ADRENALINE AND NORADRENALINE, RATE OF URINE SECRETION, AND BLOOD PRESSURE IN CATS BEFORE, DURING, AND AFTER PIPEROXANE TEST, AN INJECTION (5 MG./KG.) AND THEN AN INFUSION (2·5 MG./KG./30 MIN.) WERE MADE

Means of six experiments. 30 min. periods.

Period	Adrenaline (ng./kg./min.)	Noradrenaline (ng./kg./min.)	Urine (ml./kg./min.)	B.P. (mm. Hg)
1 and 2	0·85	2·9	0·07	130→118
Injection				
3	1·5	4·8	0·07	97
Infusion started				
4 to 8	2·6	5·3	0·12	109→102
Infusion stopped				
9 and 10	2·6	4·2	0·16	105→110

TABLE IV

URINARY EXCRETION OF ADRENALINE AND NORADRENALINE, RATE OF URINE SECRETION, AND BLOOD PRESSURE IN CATS BEFORE, DURING, AND AFTER HYDERGINE INJECTIONS

Means of four experiments. 30 min. periods.

Period	Adrenaline (ng./kg./min.)	Noradrenaline (ng./kg./min.)	Urine (ml./kg./min.)	B.P. (mm. Hg)
1 and 2	0·30	1·6	0·07	139→103
Hydergine (1 mg./kg.)				
3 and 4	1·9	1·1	0·09	94→108
Hydergine (1 mg./kg.)				
5 to 10	0·97	0·38	0·13	105→80

TABLE V

URINARY EXCRETION OF ADRENALINE AND NORADRENALINE, RATE OF URINE SECRETION, AND BLOOD PRESSURE IN CATS BEFORE, DURING, AND AFTER HYDRALLAZINE INJECTIONS

Means of five experiments. 30 min. periods. Injections of hydergine (1–5 mg./kg.) were given intravenously as needed.

Period	Adrenaline (ng./kg./min.)	Noradrenaline (ng./kg. min.)	Urine (ml./kg./min.)	B.P. (mm. Hg)
1 and 2	0·75	1·4	0·08	154→133
Injections started				
3 to 8	2·5	1·9	0·09	86→111
Injections stopped				
9 and 10	3·9	2·6	0·12	108→90

under similar conditions contained a mean of 4.2 ng./ml. of adrenaline and only 16.6 ng./ml. of noradrenaline (Benfey, Mazurkiewicz, and Melville, 1958b).

We have repeated, with certain modifications, experiments in which dogs were infused with noradrenaline before and after phenoxybenzamine (Benfey *et al.*, 1959). Hexamethonium was given at 0.1 mg./kg./min. throughout the experiment in order to eliminate an action of phenoxybenzamine on the excretion of endogenous noradrenaline. After a control period of 1 hr., 0.2 μg./kg./min. of noradrenaline was infused for 1 hr.; phenoxybenzamine was injected 1 hr. later. A second infusion of noradrenaline was started 1 hr. after phenoxybenzamine.

The clearances of *p*-aminohippuric acid and creatinine before and after phenoxybenzamine were also determined (Table VI and Fig. 2). The control values for noradrenaline (periods 1, 2, and 6 to 8) were low. Before phenoxybenzamine, the noradrenaline excretion in periods 3 to 5 was 290 ± 40 and after phenoxybenzamine in periods 7 to 9 it was 454 ± 49 ng./kg. $\pm$S.E. The difference was statistically significant ($P < 0.05$). Taking into account the control urinary noradrenaline output of 0.4 ng./kg./min. (Table VI), the urinary recovery of infused noradrenaline was 2.1% before and 3.5% after phenoxybenzamine. The urine output was 10.0 ml./kg. during periods 3 to 5 and 10.6 ml./kg. during periods 7 to 9. The mean *p*-aminohippuric acid clearance was 12.2 ml./kg./min. in periods 3 and 4 and 10.7 in periods 7 and 8, and the creatinine clearance was also somewhat reduced from 5.7 ml./kg./.min. during periods 3 and 4 to 4.7 during periods 7 and 8. A change in "kidney function" was therefore not responsible for the effect of phenoxybenzamine. There was no evidence that the infusion of noradrenaline raised the urinary excretion of adrenaline, so there was no indication that part of the infused noradrenaline might have been converted to adrenaline.

DISCUSSION

Our experiments have shown that phenoxybenzamine raised the urinary excretion of noradrenaline in cats only when there was hypotension. Its action was indirect and secondary to a reflex stimulation of sympathetic tone. Hypotension in the absence of antisympathomimetic agents had little influence on the excretion of noradrenaline. It has previously been shown in dogs that hypotension in the early stages of haemorrhage (Millar and Benfey, 1958) or after repeated injections of methacholine (Benfey *et al.*,

TABLE VI

URINARY EXCRETION OF ADRENALINE AND NORADRENALINE, BLOOD PRESSURE, URINE SECRETION, CLEARANCE OF *P*-AMINOHIPPURIC ACID, AND OF CREATININE, AND FILTRATION FRACTION IN DOGS DURING INFUSION WITH NORADRENALINE BEFORE AND AFTER PHENOXYBENZAMINE INJECTION

Intravenous infusion of sodium chloride (0.9%) and hexamethonium (0.05%) was given throughout the experiment (0.2 ml./kg./min.). A, indicates adrenaline (ng./kg./min.); N, noradrenaline (ng./kg./min.); B.P., blood pressure in mm. Hg; U, urine secretion (ml./kg./min.); C_{PAH}, clearance of *p*-aminohippuric acid (ml./kg./min.); C_{CR}, clearance of creatinine (ml./kg./min.); FF, filtration fraction. Noradrenaline infusion, 0.2 μg./kg./min. Phenoxybenzamine injection 10 mg./kg. Means of four experiments. 30 min. periods.

Period	A	N	B.P.	U	C_{PAH}	C_{CR}	FF
1 and 2	1·3	0·30	117→112	0·107	33·3→13·1	14·0→5·9	0·461
Noradrenaline infusion started							
3 and 4	1·2	4·1	119→120	0·121	12·2	5·7	0·478
Noradrenaline infusion stopped							
5 and 6	1·0	1·6→0·30	109	0·095			
Phenoxybenzamine injection							
7 and 8	1·0	0·45	109→97	0·113	8·2	4·1	0·523
Noradrenaline infusion started							
9 and 10	1·1	6·3	92	0·128	10·7	4·7	0·461
Noradrenaline infusion stopped							
11	0·50	2·7	88	0·098			

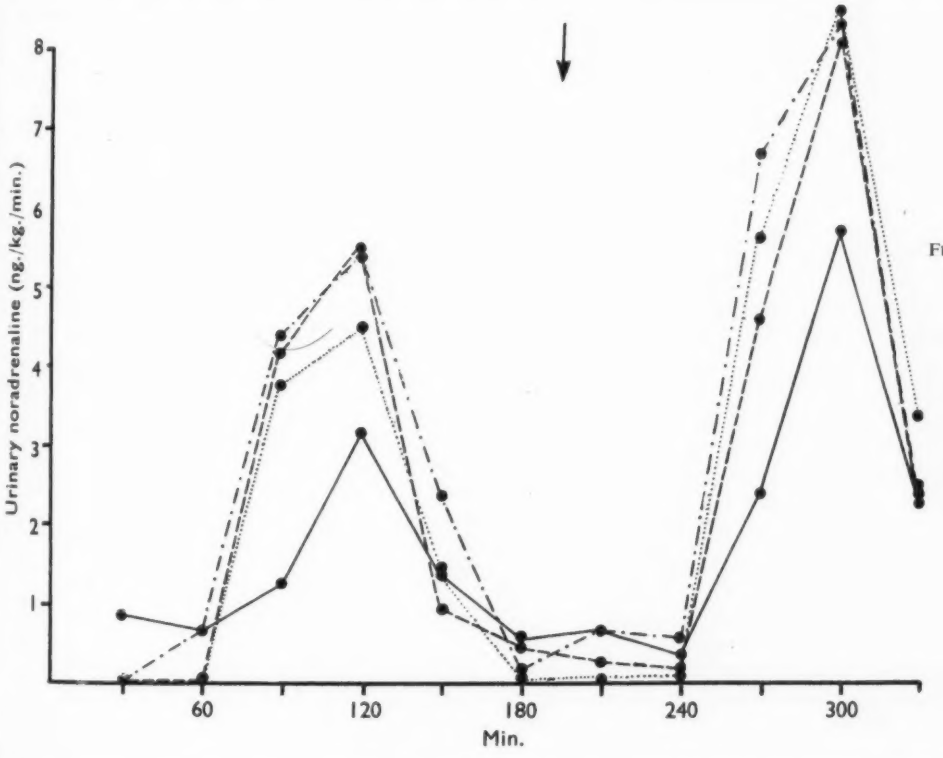

FIG. 2.—Urinary excretion of noradrenaline before and following administration (at the arrow) of phenoxybenzamine (10 mg./kg.). Each line represents a record from an individual dog. Noradrenaline (0.2 μg./kg./min.) was infused from 61 to 120 and 241 to 300 min.

1959) raises mainly the urinary excretion of adrenaline. In the present experiments, hydrallazine, of which the main action appears to be directly on the blood vessels (Moyer, Huggins, and Handley, 1953) and which possesses slight if any anti-sympathomimetic action, raised the noradrenaline excretion only a little and not greatly above that of adrenaline as did the other antagonists. It was difficult to find an agent which lowered the blood pressure for a prolonged period of time, was free from action on sympathetic ganglia or receptors and did not possess side actions which made it unfit for purposes of comparison.

The increase in urinary noradrenaline after phenoxybenzamine thus appeared to be accounted for satisfactorily by the contention of Brown and

Gillespie (1957) that "the mechanism for the destruction of noradrenaline at the nerve endings is linked to the receptors for this substance and can be inactivated with them." These workers found that, by giving phenoxybenzamine, they could raise the noradrenaline concentration in the venous outflow from the spleen when stimulating the splenic nerves in cats. It may be assumed that, in the present experiments, hypotension activated sympathetic fibres reflexly and liberated noradrenaline which, in the presence of antisympathomimetic drugs, appeared in the urine in higher concentrations because destruction was impaired. We have previously shown that phenoxybenzamine increased the urinary output of noradrenaline when large doses of acetylcholine were repeatedly given after atropine (Benfey *et al.*, 1959) and, further, phenoxybenzamine raised the plasma concentration of noradrenaline in dogs and increased the plasma noradrenaline concentration in adrenalectomized dogs subjected to haemorrhage (Millar *et al.*, 1959).

Phenoxybenzamine and dibenamine appeared to be the only antisympathomimetic drugs that were reasonably free from side actions. Piperoxane and phentolamine have been reported to stimulate the sympathetic nervous system by central action (Handovsky, 1935; Gross, Tripod, and Meier, 1951). Their effect on the urinary excretion of adrenaline and noradrenaline might therefore be due partly to direct sympathetic stimulation. In addition, their hypotensive action was not very strong. Hydergine, on the other hand, is known to depress the vasomotor centre, so reducing the blood pressure (Konzett and Rothlin, 1953). This might explain our divergent result with this drug, which moderately reduced blood pressure but did not elevate the urinary excretion of noradrenaline.

There were great variations in the urinary excretion of adrenaline and noradrenaline in the control as well as in the experimental periods. Generally, the control values in cats were higher than in dogs. This might have been due to the induction of anaesthesia with ether which is known to stimulate the sympathetic nervous system. This was followed by sympathetic depression during anaesthesia with pentobarbitone. The latter was generally deep but not controlled, and it is conceivable that the state of the animals (particularly the responsiveness of their sympathetic system) varied considerably. We have tried to relate the blood pressure to the adrenaline and noradrenaline output in the control period, but this proved impossible. A low blood pressure was often but not always associated with high urinary excretion of adrenaline and noradrenaline.

Hexamethonium appeared to exert a stabilizing influence; the blood pressure was well maintained after phenoxybenzamine and the urinary excretion of adrenaline and noradrenaline was uniformly low.

Studies of the action of antisympathomimetic drugs on the destruction of adrenaline and other catechol amines are in progress. In the present experiments the behaviour of adrenaline has been rather puzzling. The urinary excretion of adrenaline was reduced by phenoxybenzamine but increased by hydrallazine and the other anti-adrenaline substances.

The authors are indebted to Professor K. I. Melville for his interest in this work, which was supported by a grant from the National Research Council of Canada.

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A HYPOTHESIS CONCERNING THE EFFECT OF COCAINE ON THE ACTION OF SYMPATHOMIMETIC AMINES

BY

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(RECEIVED MAY 15, 1959)

There is a store, perhaps in chromaffin tissue, of noradrenaline in tissues with a sympathetic innervation. The store is depleted by treatment with reserpine. Sympathomimetic amines like tyramine act only when this store is present and have no effect when the store is depleted. They also fail to act in the presence of cocaine. Catecholamines like noradrenaline have a much greater action than usual when the store is depleted, and they have a much greater action in the presence of cocaine. It is suggested that cocaine has the effect of blocking release from the store, so that the action of tyramine is abolished and the spontaneous release from the store which is responsible for the normal (low) sensitivity to noradrenaline is stopped. Noradrenaline is taken up into the store in the heart and the vessel wall from the blood, and the disappearance of noradrenaline from the blood is in part due to this uptake and not entirely to destruction. Cocaine may prevent this uptake of noradrenaline by the tissue stores. Experiments on rabbit atria, on the vessels of the rabbit ear and on the heart-lung preparation are described which are consistent with this hypothesis.

An early observation on cocaine was made by Fröhlich and Loewi (1910), who found that it potentiated the action of adrenaline. Tainter and Chang (1927) found that cocaine affected the action of tyramine in the opposite way, reducing or abolishing it. Recently the observation has been made that similar changes occur in animals treated with reserpine; the effects of adrenaline and noradrenaline are potentiated (Bein, Gross, Tripod and Meier, 1953) while the effect of tyramine is abolished (Carlsson, Rosengren, Bertler and Nilsson, 1957). Burn and Rand (1958b) showed that treatment with reserpine removed the store of noradrenaline from the vessel wall and suggested that, while the effects of reserpine were due to the disappearance of the store, cocaine might act by stopping release from the store (Burn and Rand, 1958c); the action of noradrenaline would then be potentiated because more receptors would be free for injected noradrenaline to occupy. Similarly if release from the store was stopped, tyramine would be ineffective. Observations have now been made to test this view of the action of cocaine.

These have been made first on the rate of the isolated atria of the rabbit heart, in which Burn and Rand (1958a) found that when the store of noradrenaline was present the rate was higher. Observations have also been made in the perfused rabbit ear and in the heart-lung preparation of the dog.

METHODS

Rabbit atria were suspended in a 40 ml. bath containing McEwen (1956) solution at 30° aerated with O_2 and 5% CO_2. The upper end of the atria was attached to a spring lever carrying a wire which dipped into a salt solution so that, with every contraction, a circuit containing a post office counter was completed. This recorded atrial beats, which were expressed as beats/minute. When treating rabbits with reserpine, the drug was prepared in a 20% solution of ascorbic acid. Rabbits weighing 2.0 to 2.5 kg. received 3.0 to 5.0 mg. of reserpine intraperitoneally on the first and second day, the amount given on the second day depending on the general condition of the animal. On the third day the rabbit was decapitated, and the atria were removed. Observations were also made by perfusing the vessels of the rabbit ear and recording the outflow with a recorder (Stephenson, 1948). Locke solution was used as the perfusion fluid. It was aerated with O_2 before being placed in the Marriotte bottle. The perfusions were carried out at room temperature. Observations were also made in the heart–lung preparation of the dog. The blood used to fill the reservoir was taken from

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one dog which was anaesthetized with ether; the preparation was then made in a second dog which was first anaesthetized with ether and then with chloralose. The heart rate was counted from an electrocardiogram recorder (Cossor model 1314).

The weights of cocaine refer to the hydrochloride.

Results

Effect of Cocaine on the Atrial Rate.—When normal atria were exposed to cocaine hydrochloride for periods up to 30 min., no change in atrial rate was observed until the concentration was raised to 7.5×10^{-6} or to 10^{-5} g./ml. A decrease in rate was then observed in the presence of cocaine, the rate recovering its former value when the cocaine was removed from the bath. In some experiments (see Fig. 1), the drop in rate in the presence of cocaine became progressively

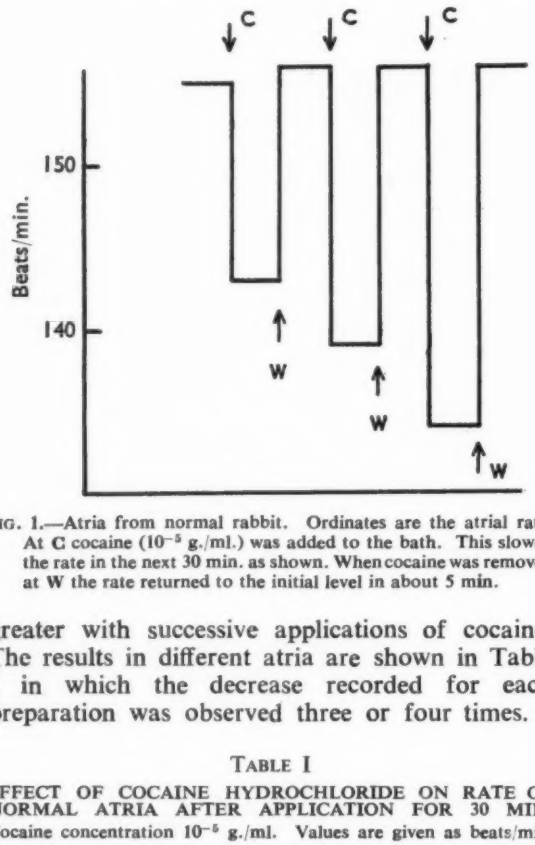

FIG. 1.—Atria from normal rabbit. Ordinates are the atrial rate. At C cocaine (10^{-5} g./ml.) was added to the bath. This slowed the rate in the next 30 min. as shown. When cocaine was removed at W the rate returned to the initial level in about 5 min.

greater with successive applications of cocaine. The results in different atria are shown in Table I in which the decrease recorded for each preparation was observed three or four times.

TABLE I

EFFECT OF COCAINE HYDROCHLORIDE ON RATE OF NORMAL ATRIA AFTER APPLICATION FOR 30 MIN. Cocaine concentration 10^{-5} g./ml. Values are given as beats/min.

Expt. No.	Decrease of Rate in Successive Trials
1	10, 8, 8
2	12, 16, 18, 18
3	6, 13, 10, 11
4	9, 12, 11, 6
5	12, 17, 22
6	11, 22, 35

Effect of Cocaine on Atria from Rabbits Treated with Reserpine.—When the atria were removed from rabbits treated with reserpine, their rate of beating was lower than that of atria from normal rabbits, the mean rate being 115/min. compared with 130/min. When the atria were exposed to cocaine for 30 min., the effect was not the same in all preparations. In 4 in which the spontaneous rate was low, namely 114/min., and in which reserpine appeared to have exerted a full effect, the rate was depressed in the presence of cocaine, but was not restored when the cocaine was removed. An example of such a preparation is given in Fig. 2. In one other preparation, there was a partial restoration on washing out the cocaine, but, as the experiment progressed, the restoration was less and less complete. Finally in 2 in which the spontaneous rate was high, namely 140/min., the rate was depressed in the presence of cocaine, and was restored when it was washed out as in normal atria.

Potentiation of Adrenaline by Cocaine.—Evidence that cocaine potentiates the effect of adrenaline on the isolated heart of the cat was obtained by Burn and Tainter (1931), but there is no previous evidence that cocaine potentiates the action of adrenaline on the atria. Adrenaline in a concentration of 10^{-7} g./ml. caused a rise of rate of 10 to 30 beats/min. when allowed to act for 10 min., and this effect was found to be increased in the presence of cocaine hydrochloride 10^{-6} g./ml. An example of an experiment is given in Table II. When an observation

TABLE II

INCREASE OF ATRIAL RATE BY ADRENALINE AND ITS POTENTIATION WHEN COCAINE WAS ADDED 10 MIN. PREVIOUSLY

The values are increase in beats/min. observed consecutively in one experiment. Adrenaline concentration, 10^{-7} g./ml.; cocaine concentration, 10^{-6} g./ml.

Adrenaline	30	
Adrenaline + cocaine		43
Adrenaline	11	
"	10	
Adrenaline + cocaine		50
Adrenaline	31	
Adrenaline + cocaine		35
Adrenaline	14	
Adrenaline + cocaine		46
	19	43

was to be made in the presence of cocaine, it was first added to the bath and time was allowed to ensure that it did not modify the rate before adrenaline was added. Table II shows that in the presence of cocaine the mean increase in rate caused by adrenaline was more than twice that in the absence of cocaine. The mean effects in three experiments are given in Table III.

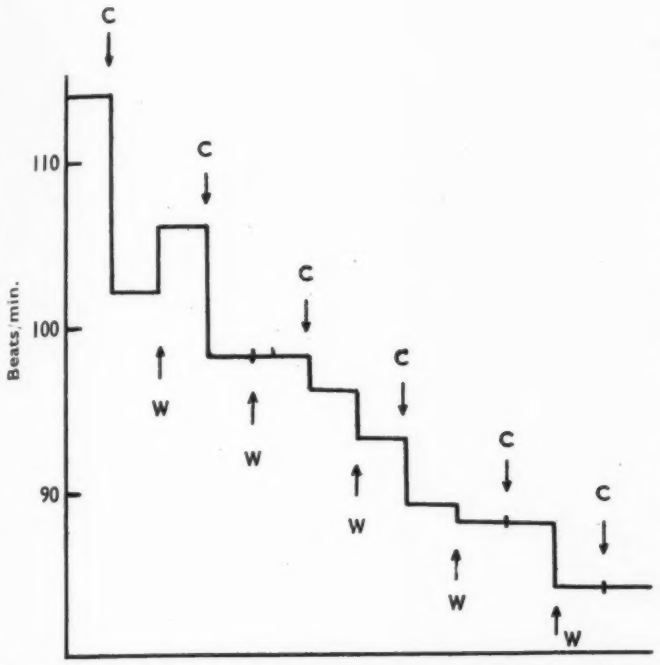

FIG. 2.—Atria from a rabbit treated with reserpine. Ordinates are the atrial rate. Note that, although the addition of cocaine (C) slowed the rate, removal of the cocaine (W) did not restore the rate except partly after the first addition.

Potentiation in Atria from Rabbits Treated with Reserpine.—The effect of adrenaline alone was greater in these atria. Thus the mean increase in rate caused by adrenaline (Table III, Expt. nos. 1, 2, and 3) was 33 beats/min., while the mean increase in rate caused by the same concentration of adrenaline in atria from rabbits treated with reserpine (Table IV) was 62 beats/min. Table IV also shows that the potentiation of the effect of adrenaline by cocaine was almost absent in three of the atria, and small in the fourth.

Similar experiments were carried out with noradrenaline on atria from rabbits treated with reserpine. Noradrenaline had more effect in increasing the rate of these atria (mean increase 69 beats/min.) than it had in increasing the rate of normal atria (mean increase 52). As with adrenaline, the potentiation of the effect of noradrenaline by cocaine was much less than in the normal atria, the mean effect of cocaine in 4 out of 5 experiments being very small.

Potentiation of Noradrenaline by Cocaine.—In other atria the action of noradrenaline was examined. Whereas adrenaline was tested in a concentration of 10^{-7} g./ml., noradrenaline was applied in half this concentration, and it appeared to cause a greater rise in the rate. The action of noradrenaline was clearly potentiated by cocaine as the mean results of three experiments in Table III show.

Potentiation by Cocaine in Vessels of Rabbit Ear.—Experiments were carried out to observe whether cocaine potentiated the constrictor action of noradrenaline and of adrenaline in the vessels of the perfused rabbit ear. It was found that cocaine regularly caused potentiation, as shown in Fig. 3 in which 0.002 μg. of noradrenaline was

TABLE III

MEAN RESULTS SHOWING INCREASES IN ATRIAL RATE CAUSED BY ADRENALINE AND BY NORADRENALINE AND THE AUGMENTED INCREASES IN THE PRESENCE OF COCAINE

Concentrations: adrenaline, 10^{-7} g./ml.; noradrenaline, 5×10^{-8} g./ml.; cocaine, 10^{-6} g./ml. The rates are given as beats/min.

Expt. No.	Adrenaline	Adrenaline and Cocaine	Potentiation
1	19	43	2·3
2	60	73	1·2
3	19	35	1·8
Mean	33	50	1·8
	Noradrenaline	Noradrenaline and Cocaine	
4	41	76	1·85
5	27	75	2·8
6	87	104	1·2
Mean	52	85	1·95

TABLE IV

MEAN RESULTS SHOWING INCREASES IN ATRIAL RATE CAUSED BY ADRENALINE AND BY NORADRENALINE IN ATRIA FROM RESERPINE-TREATED RABBITS AND THE EFFECT OF COCAINE

Concentrations: adrenaline, 10^{-7} g./ml.; noradrenaline, 5×10^{-8} g./ml.; cocaine, 10^{-6} g./ml. The rates are given as beats/min.

Expt. No.	Adrenaline	Adrenaline and Cocaine	Potentiation
7	66	73	1·1
8	68	97	1·4
9	43	43	1·0
10	71	75	1·0
Mean	62	72	1·1
	Noradrenaline	Noradrenaline and Cocaine	
11	50	86	1·7
12	77·5	94	1·2
13	85	93	1·1
14	85	79	0·93
15	46	58	1·2
Mean	69	82	1·2

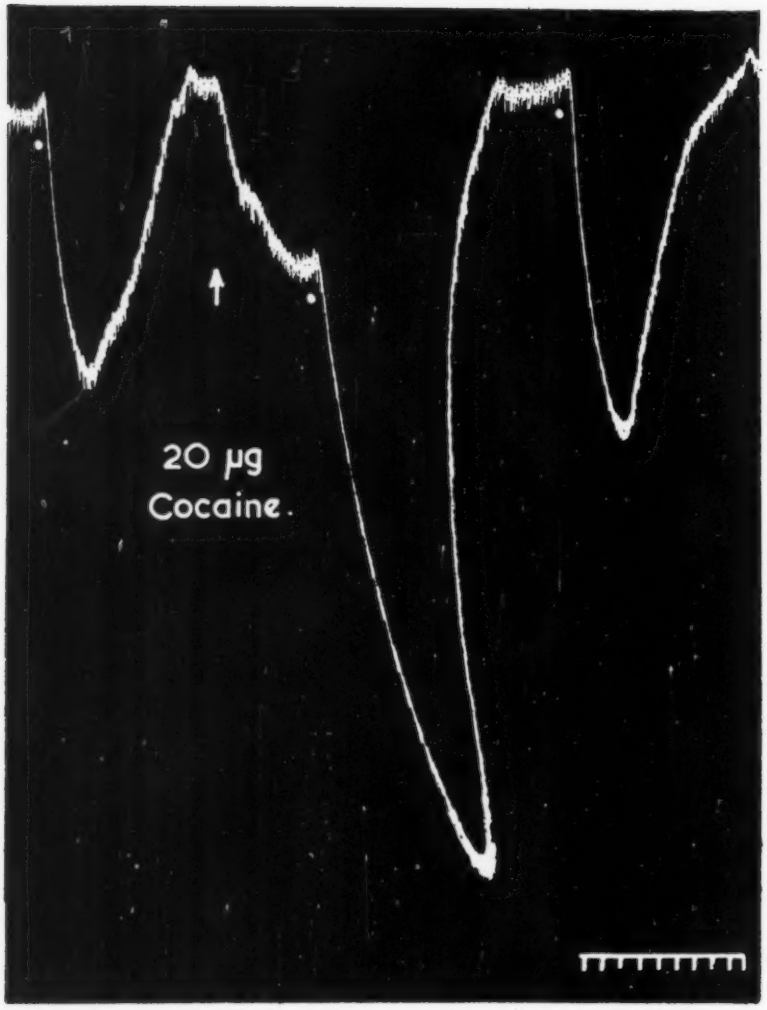

FIG. 3.—Record of venous outflow from rabbit ear perfused with Locke solution. At the white dots an injection of 0·002 µg. of noradrenaline was made into the artery cannula. Before the second of these injections 20 µg. cocaine was injected at arrow. This increased the effect of noradrenaline. Time, min.

injected three times. The second injection was preceded by the injection of 20 µg. of cocaine hydrochloride, and noradrenaline then caused much greater vasoconstriction. Similar results were obtained with adrenaline.

Observations in the Heart-Lung Preparation.—Tyramine causes an increase in rate in the heart-lung preparation and Bejrablaya, Burn and Walker (1958) have already shown that in the presence of cocaine the effect was greatly reduced. Since they showed that tyramine had almost no effect on the rate in a preparation made from a dog previously given reserpine, they concluded that tyramine must act by liberating noradrenaline. The effect of giving cocaine has now been tested, not before the injection of tyramine, but after the injection when the rise in rate had reached its peak. Fig. 4 shows the increase in rate in one experiment in which 1 mg. of tyramine hydrochloride was injected into the superior vena cava; the increase lasted for 25 min. The injection of tyramine was then repeated, and 1 min. later 20 mg. of cocaine was injected. The rate fell rapidly and was back to the initial level in 8 min.

Cocaine and Reserpine.—An exhaustive study of the action of reserpine in the heart-lung preparation has been made by Krayer and Fuentes (1958) and Paasonen and Krayer (1958), who have followed the rise in heart rate and the diminution in the amount of noradrenaline in the cardiac tissue. Some experiments have been made to see how the effect of reserpine on the heart rate was modified when cocaine was given beforehand and when it was given during the action of reserpine. Fig. 5 shows the rise in heart rate in an experiment (**A**) in which 1 mg. of reserpine was added to the venous reservoir, no cocaine being given, and also the rise in an experiment (**B**) when 20 mg. of cocaine was added to the venous reservoir 25 min. before the addition of 1 mg. of reserpine. In this experiment it seemed clear that the presence of cocaine almost abolished the rise in rate which reserpine would have caused. The effect of cocaine in other experiments was less, as shown in Table V,

TABLE V

EFFECT OF 1 MG. RESERPINE ON THE ATRIAL RATE WHEN ADDED TO RESERVOIR IN HEART-LUNG PREPARATION IN THE DOG. COCAINE (20 MG.) WAS ADDED 15–20 MIN. PREVIOUSLY IN THE LAST THREE EXPERIMENTS

Rates are given as beats/min.

Initial Rate	Maximum Rate	Maximum Increase in Rate	Total Area of Increase Determined Graphically
156	203	47	446
137	181	44	324
126	162	36	160
169	202	33	202
169	173	4	57

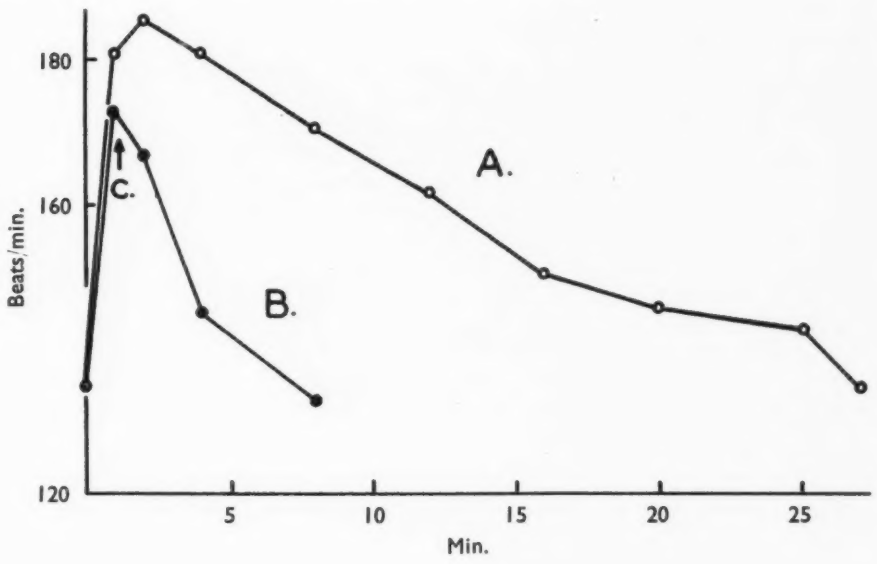

FIG. 4.—Records of heart rate taken from dog heart-lung preparation. Ordinates: beats/min. Abscissae: time in min. **A** shows effect of injecting 1 mg. of tyramine. **B** shows the effect of repeating this injection, but adding 20 mg. of cocaine (**C** at arrow) to the reservoir 1 min. later.

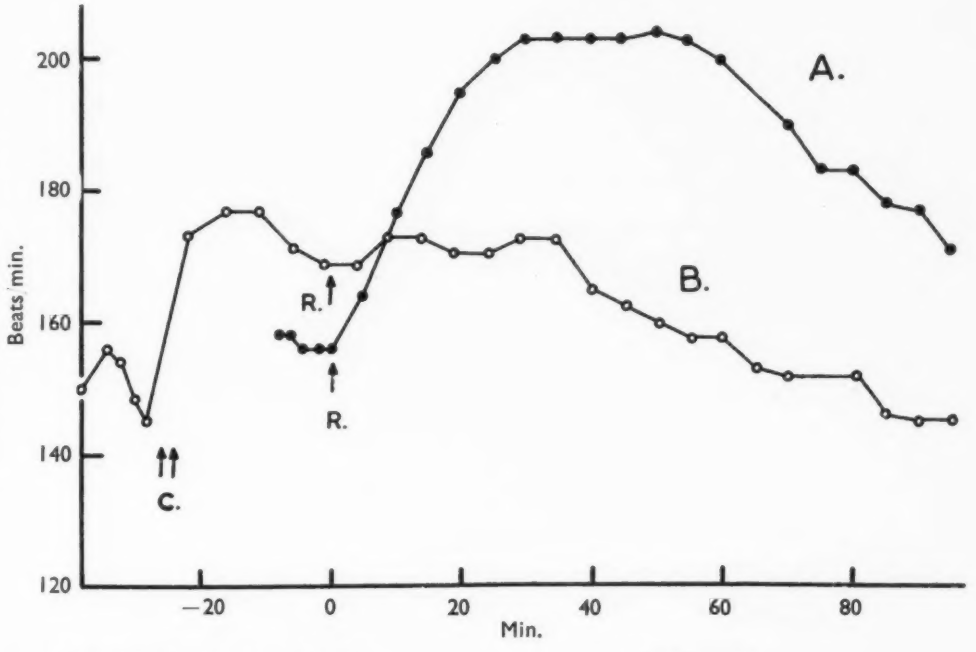

FIG. 5.—Records of heart rate from a dog heart-lung preparation. **A** shows effect of adding 1 mg. of reserpine (**R**) to the reservoir. **B**, taken in another experiment, shows the effect of adding 10 mg. of cocaine at each arrow (**C**) to the reservoir, and then of adding 1 mg. of reserpine (**R**) to the reservoir.

but it appeared to reduce the rise in rate both in height and duration. The effect on the rate was recorded not only as the maximum increase, but as the total area of increase obtained by plotting the rate on squared paper.

When reserpine was given first, and the addition of cocaine was made 44 min. later, the cocaine caused a large rise in rate as shown in Fig. 6. Further additions of cocaine were made as shown in Fig. 6, but it is not clear whether they had any action.

DISCUSSION

The observations may now be considered in the light of the hypothesis concerning the action of cocaine which was mentioned in the introduction. Burn and Rand (1958a) found that normal atria isolated in the organ bath contracted at a mean rate of 146/min., while atria from rabbits treated with reserpine contracted at a mean rate of 112/min. They concluded that the noradrenaline present in the normal atria was responsible for the higher rate and that there must always be a small

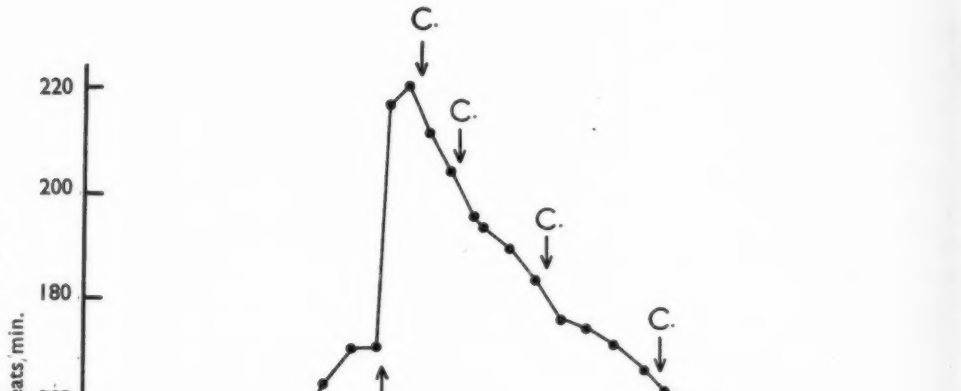

FIG. 6.—Record of heart rate from a dog heart-lung preparation. At R, 1 mg. of reserpine was injected into the superior vena cava. At C, 10 mg. of cocaine was injected. Note the increase in rate caused by the first injection of cocaine.

discharge of noradrenaline from the store. The hypothesis suggested that cocaine stopped this discharge, and in the present experiments it was found that cocaine reduced the rate of beating sometimes by as much as 35 beats/min., the rate returning to the previous level on washing out the cocaine. Furthermore, this action of cocaine was not observed in the majority of atria from rabbits treated with reserpine. In such atria there was no store of noradrenaline. When cocaine was added to the bath there was some fall in rate, but, on washing out the cocaine, the rate did not return to the initial level ; the rate fell progressively with further additions of cocaine. There is no evidence to explain this progressive fall, but it may have been due to some other depressant action made evident in atria treated with reserpine, or even to a spontaneous fall.

The increase in atrial rate caused by adding adrenaline or noradrenaline to the bath was greater in the presence of cocaine, the effect of cocaine being almost to double the increase. This action of cocaine can be interpreted as indicating that cocaine not only prevents a discharge of noradrenaline from the store but that it also prevents the uptake of noradrenaline by the store. Evidence that cardiac tissue takes up noradrenaline was described by Raab and Gigee (1955), but it depended on the administration of amounts of noradrenaline or of adrenaline which were far beyond the physiological range. They injected 10 mg./kg. intraperitoneally, and this for a dog of 10 kg. would be a dose of 100 mg. They observed, for example, that after the injection, noradrenaline rose from 0.202 μg./g. heart muscle to 1.16 μg./g., and, when a mixture of noradrenaline and adrenaline was injected, to a value of 2.4 μg./g. Later Euler (1956) gave noradrenaline by intravenous infusion as well as by intraperitoneal injection in the cat. He infused amounts up to 7.8 μg./kg./min. for 30 min. and injected mixtures of noradrenaline and adrenaline up to 2 mg./kg. into the peritoneal cavity. He found that these amounts did not significantly alter the catechol amine content of the heart, spleen, liver, kidney or skeletal muscle, when prepared 5 to 28 min. after the end of the infusion.

Evidence of a different kind that noradrenaline was taken up from the blood by artery walls, the nictitating membrane and the spleen has been obtained by Burn and Rand (1958c), and that it was taken up by the heart has been obtained by Bejrablaya *et al.* (1958). These workers found that the action of tyramine was absent when the

animal had been treated with reserpine. Since this treatment was shown to discharge the store of noradrenaline in the blood vessels (Burn and Rand, 1958b), the spleen (Burn and Rand, 1959) and the heart (Bertler, Carlsson and Rosengren, 1956), it was concluded that tyramine acted by liberating noradrenaline. It was found that an infusion of noradrenaline into the spinal cat restored the action of tyramine on the blood pressure, the nictitating membrane, and the spleen, and a similar infusion into the heart-lung preparation increased the duration of the action of tyramine on the heart rate.

Recently Gillespie and Mackenna (1959) have obtained evidence that noradrenaline and adrenaline can be taken up by a tissue in an isolated organ bath. They have made observations by stimulating the lumbar colonic nerves and have recorded relaxation of the colon. When the preparation was made from an animal previously given reserpine, they found that sympathetic stimulation caused contraction and not relaxation, but that after the addition of noradrenaline or of adrenaline to the bath, then after 15 min. or more stimulation once more caused relaxation.

These results suggest that when noradrenaline is put into the blood stream or into an isolated organ bath, some of it acts on sympathetic receptors producing its usual effect, while some of it is taken up by the store. If cocaine prevents the uptake by the store, then in the presence of cocaine the whole amount will act on the sympathetic receptors. The observations of Trendelenburg (1959) support this. He has studied the rate of disappearance of noradrenaline from the blood of the spinal cat after the injection of 25 μg./kg. and found that it was greatly reduced in the presence of cocaine, and that the effect on the blood pressure was correspondingly increased.

The potentiation by cocaine of the action of noradrenaline and of adrenaline in atria, and also in the perfused rabbit ear, may therefore be due to cocaine blocking the uptake by the store. The greater effect of noradrenaline and adrenaline in atria from rabbits treated with reserpine and the much smaller potentiating action of cocaine may be due to the action of reserpine which, having depleted the store, presumably by competing for and occupying the storage receptors, prevents relatively small concentrations of noradrenaline from displacing it from these receptors.

The evidence obtained from the experiments in the heart-lung preparation indicated that cocaine was able to cut short the release of noradrenaline from the store by the action of tyramine. There was also evidence that it could reduce the effect of reserpine, provided that the cocaine was given in large doses beforehand. If, however, reserpine was given first, the effect of cocaine was to potentiate the action of the noradrenaline which the reserpine was releasing. If the hypothesis put forward is correct, it is possible that some of the noradrenaline released by reserpine was being taken up again by the store, and that cocaine prevented this uptake.

In conclusion it is scarcely necessary to say that we are dealing here with new conceptions. The evidence for the existence of a store possibly present in chromaffin tissue, from which noradrenaline can be discharged and by which it can be taken up, is already considerable. But the mechanisms involved are unknown and hypotheses concerning them can only be tentative.

This work was done during the tenure of a Special Research Fellowship awarded by the National Heart Institute, U.S.P.H.S. I wish to thank Professor J. H. Burn for suggesting this problem to me and for his guidance throughout the work. I also wish to thank Miss Roneen Hobbs for making the observations on the rabbit ear.

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THE CARDIO-ACTIVE PRINCIPLE IN SPLEEN

BY

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A substance has been demonstrated in acetone extracts of freeze-dried spleen which had cardiotonic properties on isolated papillary muscles from the cat and perfused hearts of the cat and guinea-pig. This activity of the extract could not be explained by the presence of choline, acetylcholine, histamine, 5-hydroxytryptamine, adrenaline or noradrenaline.

The progressive deterioration of a heart-lung preparation has been shown to be abolished by the introduction of the liver into the circuit (Bassani, 1933; Pinotti, 1942; Rein, 1942). These authors demonstrated that glucose, bile ingredients or sympathomimetic amines could not be responsible for the tonic effect of the liver, and Rein (1942) postulated that, since the tonic effect resembled that of the cardiac glycoside strophanthin, the liver might add a steroid to the blood.

Subsequently, Rein, Mertens, and Bücherl (1949) demonstrated a spleen-liver regulatory system which protected the organism as a whole, and the heart specifically against oxygen lack by regulating oxidative metabolism. Under conditions of cardiac hypoxia, Bücherl and Rein (1949) found that the spleen released a substance (*hypoxie-l'enen*) which was transported by the blood stream to the liver, where it stimulated the release of an active material resembling strophanthin since it caused greater economy of cardiac work. Rein (1951) found that transhepatic administration of splenic venous blood from a donor to a recipient animal with acute cardiac hypoxia overcame progressive cardiac failure.

Extracts of liver were shown by Green (1952) to have a pronounced stimulant effect upon isolated papillary muscle of the cat. Green and Nahum (1957) examined commercial liver extracts for cardiotonic activity and found some contained tyramine, although the presence of this substance was thought to be due to bacterial contamination.

Stimulant actions of splenic extracts upon the heart have been attributed by Rothlin (1920) to histamine which was subsequently isolated from spleen (Dale and Dudley, 1929). We have previously shown that extracts of spleen can stimulate isolated cat papillary muscle (Cobbin and Thorp, 1957). The purpose of the present paper is to show that this activity cannot be due to histamine, adrenaline, noradrenaline, acetylcholine, choline, or 5-hydroxytryptamine, all of which occur in splenic tissue.

METHODS

Preparation of the Splenic Extracts

Three separate extractions of spleen were performed.

Extraction (1).—Ox spleen (3.37 kg.) from the abattoir was decapsulated, passed through a hand mincer and freeze-dried. A sample was extracted in a Soxhlet apparatus with the following purified solvents, first petroleum ether, then ether and finally acetone. The acetone fraction was evaporated to dryness and yielded a dark brown tarry residue (2.37 mg./1.0 g. of fresh spleen).

Extraction (2).—A freeze-dried preparation from 17 kg. of ox spleen by *Extraction* (1) was powdered in a mortar, transferred to a Soxhlet apparatus and extracted with anhydrous ether. A cold extraction with anhydrous acetone followed, the acetone was removed under reduced pressure and the residue was taken up in distilled water. Impurities were extracted first with cold ether and then with light petroleum ether (40 to 80°). The aqueous solution was then evaporated to dryness under reduced pressure and stored below 3°. The yield was 0.33 mg. from 1 g. fresh spleen. Although this procedure gave a less potent product than *Extraction* (1), the extract was readily water soluble, there being less contamination by fat.

Extraction (3).—Ox spleen (22.5 kg.) was freeze-dried as in *Extraction* (1) and extracted in a Soxhlet apparatus first with ether and then with acetone. The acetone extract was evaporated to dryness under reduced pressure. The residue was dissolved in petroleum ether and extracted with water until no more coloured material appeared in the water layer, which was then evaporated to dryness under reduced pressure. A yield of 1.5 mg. of water soluble material was obtained from 1 g. of fresh spleen.

Biological Test Preparations

The Blood Pressure of the Cat.—Cats were anaesthetized with pentobarbitone sodium (50 mg./kg., intra-

peritoneally). The blood pressure was recorded with a mercury manometer from the left carotid artery. Intravenous injections were made into the right saphenous vein. The splenic extract [*Extraction* (2)] was dissolved in 0.9% NaCl solution in a concentration of 5 mg./ml.

Isolated Papillary Muscle Preparation.—Cats were anaesthetized with ether. Papillary muscles were dissected from the right ventricles and mounted in a 20 ml. bath of 0.9% NaCl solution at 37°. Two other solutions were used, being the modifications (a) and (c) (Creese, 1949) of the Krebs-Hensleit formula.

The muscle was stimulated by rectangular pulses of about 1 to 5 msec. duration at a rate of 75 to 90/min. through a fine silver wire (0.005 in. diameter) lightly wound round the muscle. An indifferent electrode was placed in the fluid bathing the preparation.

The force of contraction was recorded by means of a variable-inductance-transducer attached to the spring of an isometric lever to which the upper end of the muscle was tied. The transducer was coupled to an ink recorder and the relationship between tension and pen deflection was linear. At first, the muscle developed a tension of 0.5 to 2 g. according to size with a resting tension of approximately 3 g. With the usual sensitivity setting of the apparatus a tension of 1 g. corresponded to a deflection of the myograph spring of about 0.05 mm. at the point of attachment of the muscle.

When a muscle was set up first in solution (a) it contracted quite strongly and might continue to do so for many hours. In solution (c), which differed from solution (a) in the absence of $NaHCO_3$ and a reduction of Ca, the contractions of the muscle declined quite quickly. Usually this was allowed to continue until the force of contraction fell to 20 to 30% of the value when first bathed in solution (a).

The splenic extracts were amorphous pastes and were weighed on a spatula and dissolved directly in the solution (c).

Since papillary muscles set up in this way were very sensitive to changes of temperature and *p*H, all test solutions were adjusted to the same *p*H, kept at the same temperature and aerated with O_2 in the same way as the solution in the muscle bath.

The Isolated Langendorff Heart Preparation.—The isolated hearts of kittens were arranged for perfusion by the method of Langendorff at a pressure of 30 to 50 cm. of water. Ringer-Locke solution at 37° was perfused through the hearts from a reservoir containing 30 ml. of fluid maintained at a constant level by a small non-metallic centrifugal pump. Solutions of the extract were added to the reservoir or injected through a side-arm of the cannula supporting the heart. The amplitude of contraction was recorded on a kymograph by a light lever arranged to make an electrical contact at each beat which operated an impulse timer (Thorp, 1948), thus giving a record of the heart rate. Coronary flow was measured by the outflow recorder of Andrews (1952) which was calibrated after each experiment.

Isolated Guinea-pig Intestine.—A segment of guinea-pig intestine (20 to 25 cm. from the pylorus) was removed from animals of either sex weighing between 200 to 250 g., washed and mounted in a 4 ml. bath containing Tyrode solution at 35° bubbled with 5% CO_2 and 95% O_2.

Isolated Rabbit Intestine.—Rabbit intestine (20 to 25 cm. from the pylorus) was taken from animals of either sex, washed and mounted in a 10 ml. bath containing Ringer-Locke solution at 35° gassed with 5% CO_2 and 95% O_2.

Isolated Rat Uterus.—On the day preceding the experiments, virgin female rats weighing between 150 to 180 g. were given 50 μg. of oestradiol monobenzoate intramuscularly. One horn of the uterus was suspended in a 4 ml. bath containing the solution of Gaddum, Peart, and Vogt (1949) at 29°.

Results

The Action of Splenic Extract on Papillary Muscles

Splenic extract exerted a positive inotropic action on isolated cat papillary muscle confirming the findings of Cobbin and Thorp (1957). The results of ten experiments performed with the sample prepared by *Extraction* (1) are given in Table I. The threshold dose required to produce an inotropic action was greater than the equivalent of 50 mg. of fresh tissue/ml. of solution in the bath. At a dose corresponding to 100 mg. of fresh tissue/ml., a mean increase in tension to 237% of the control tension occurred, and at higher doses a graded response was found which gave an approximately linear log dose/response relationship; only single observations were made with the higher doses. The time taken to reach the peak response varied considerably, although in general the larger the dose employed the later was the peak response and the longer the duration of the effects. Frequently experiments were ended before the control amplitude was regained.

The sample prepared by *Extraction* (2) was much less potent than that prepared by *Extraction* (1). This is probably because cold acetone gives incomplete extraction. However, positive inotropic activity was found with this batch (Table I). *Extractions* (1) and (3) gave samples of approximately the same strength (see Table I).

In order to estimate the molecular size of the substance causing the positive inotropic effect, a concentrated solution was prepared in modification (c) of Krebs-Hensleit solution and passed through a cellophane ultrafilter under pressure overnight at 2°. Tested on papillary muscle, similar doses of ultrafiltrate and of crude extract gave comparable increases in amplitude of beat. Since oxytocin could just pass through the cellophane membrane, the material responsible for cardio-activity in spleen probably had a molecular weight of less than 1,000.

TABLE I

THE EFFECTS OF THE EXTRACTS OF SPLENIC TISSUE UPON THE ISOLATED CAT PAPILLARY MUSCLE

Where more than one experiment was performed the mean values are given. See text for details of *Extractions* (1), (2), and (3).

Extraction	Dose of Extract (mg. Fresh Tissue/ml.)	No. of Experiments	Increase in Amplitude (Control = 100)	Time to Maximum Effect (min.)	Duration of Response (min.)
(1)	50	3	No change	—	—
	100	4	237	14	>60
	160	1	260	50	>70
	200	1	330	8	>90
	400	1	465	90	>120
(2)	1,000	2	138	14	60–80
	2,000	1	180	15	100–120
	4,000	5	386	19	Not recorded
(3)	33	1	208	3	28–30
	67	2	350	15	>90
	100	1	230	20	>60
	300	1	420	20	>90

The Effect of Splenic Extract on the Isolated Cat Heart

When doses of 5 mg of splenic extract were injected into the side arm of the cannula attached to the isolated cat heart, the usual response was an initial depression of the amplitude of contraction lasting approximately 30 sec., and then a slight increase in amplitude together with a moderate rise in the heart rate and decrease in coronary flow. Subsequently the flow of perfusion fluid rose slightly above the control level (Fig. 1*a*). These effects were all transient, since the volumes injected were small and would have passed rapidly through the heart

When 10 mg. of extract was added to the upper reservoir essentially the same effects were observed, although they were more prolonged (Fig. 1*b*). First the heart rate returned to its control level, then the amplitude and finally the coronary flow. With very small doses, it was sometimes possible to elicit changes in amplitude without accompanying alterations in heart rate or coronary flow.

The Action of Splenic Extract in the Anaesthetized Cat

Since splenic tissue is known to contain considerable quantities of noradrenaline and lesser amounts of adrenaline (von Euler, 1950; von Euler and Purkhold, 1951a; von Euler, 1954), doses of *Extraction* (2) were injected into anaesthetized cats to see if there was a pressor response. With constant intravenous doses, it was first shown that tachyphylaxis to repeated injections did not occur. The extract caused a considerable fall in blood pressure (Fig. 2). A diminished fall in blood pressure was found 12 to 15 min. after the administration of 0.5 mg./kg. of body weight of atropine

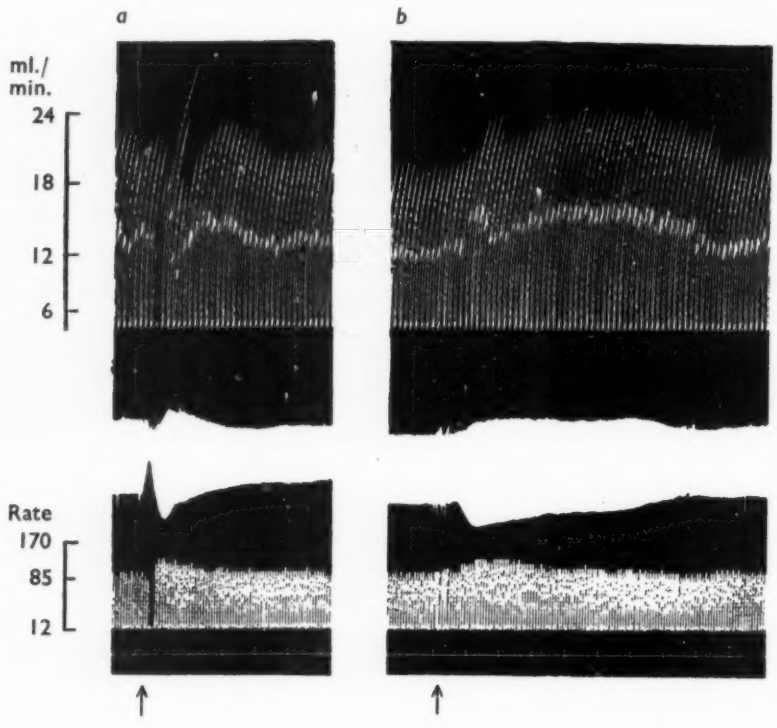

FIG. 1.—The effects of splenic extract [*Extraction* (3)] on the isolated heart of the cat. (*a*) Effect of 5 mg. crude extract injected (at arrow) into the side arm of the cannula near the entrance to the aorta. (*b*) The effect of 10 mg. crude extract added (at arrow) to the upper reservoir of fluid (vol. 30 ml.). Records from above down: coronary flow; amplitude of contraction; heart rate/min.; time, min.

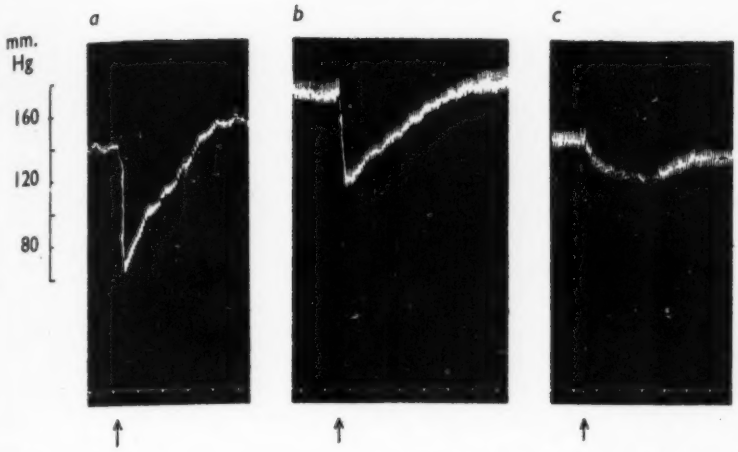

FIG. 2.—The effects of splenic extract [*Extraction* (2), 5 mg./ml. 0.9% NaCl] on the blood pressure of the anaesthetized cat. (*a*) 1 mg./kg. crude extract. (*b*) 1 mg./kg. after atropine sulphate 0.5 mg./kg. (*c*) 1 mg./kg. after promethazine hydrochloride 1.5 mg./kg. Between (*a*) and (*b*) an interval of 12 min.; between (*b*) and (*c*) an interval of 16 min. Time, min.

sulphate. Promethazine hydrochloride (1.5 mg./kg. given 15 min. previously) reduced the depressor response still further, and indeed altered the timing of the response, since the maximum fall was delayed.

Thus most of the depressor effect appeared to be due to substances resembling choline (or its esters) or histamine. The lack of a pressor response after injection of atropine and promethazine suggested the absence of sympathomimetic amines.

Exclusion of Catecholamines as the Cause of Activity in Splenic Extracts

Solutions of splenic extract in modified Krebs solution were brought to *p*H 11 ±0.1 with 4% NaOH and incubated at 37° for 1 hr. with MnO_2 and O_2 bubbled through the solution (von Euler and Purkhold, 1951b). After cooling to room temperature, the solutions were restored to *p*H 7.5 with N-HCl. Any precipitate in the alkaline solution was redissolved by the acid. The solutions were filtered to remove the MnO_2, warmed to 37° and tested on hypodynamic papillary muscles. Solutions of splenic extract not subjected to the alkaline treatment were used as controls on the same muscles.

Fig. 3 shows an experiment using doses from *Extraction* (3). It will be seen that the treated extract still shows positive inotropic activity of the same order as the untreated material. Experiments performed with *Extraction* (2) showed a similar magnitude of response for both treated and untreated extract, but the response of the incubated extract was sometimes delayed by up to 50%.

Control experiments were done with the maximum quantities of noradrenaline which might have been extracted from the spleen according to the estimates of von Euler (1954). Thus for the weakly active samples [*Extraction* (2)] the usual dose used on papillary muscles was equivalent to 3 g. fresh tissue/ml. of fluid in the 20 ml. bath. If all the noradrenaline in this quantity of spleen had appeared in the acetone extract, a total of 240 μg. would be present. After 240 μg. noradrenaline had been added to 20 ml. of modification (c) of Krebs-Hensleit solution and incubated according to the conditions described above, it had practically no action on the hypodynamic papillary muscle. This quantity of noradrenaline added without prior incubation to a papillary muscle always produced about a 300% increase in amplitude, appearing within 5 to 10 min. and thereafter decaying in an approximately linear manner. Adrenaline in similar doses was also destroyed by alkali.

To be certain that the splenic extract did not contain a substance which protected catecholamines from destruction by this procedure, 240 μg. noradrenaline was added to splenic extract and incubated as described above. When tested on papillary muscle, there was no response typical of noradrenaline, but the response to splenic extract appeared as before, reaching a maximum in 15 to 20 min. The same dose of noradrenaline and splenic extract without alkaline treatment had a strong positive inotropic action which reached a maximum in 5 to 15 min. In each experiment the

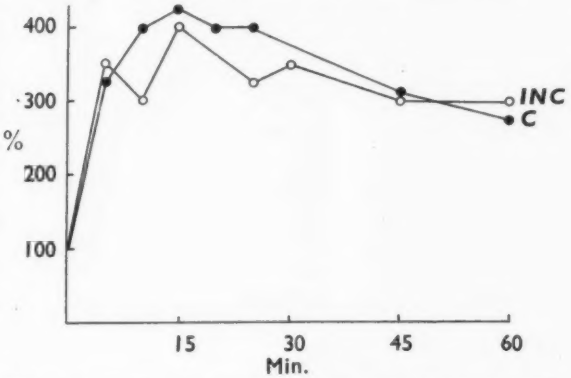

FIG. 3.—Comparison of the effects on papillary muscle of splenic extract [*Extraction* (3)]. *C*, without treatment; *INC*, after incubation at 37° at *p*H 11.0 in the presence of MnO_2 and O_2. In each instance, the dose was equivalent to 67 mg. of fresh spleen/ml. of fluid. Both experiments were performed on the same muscle, the incubated sample being tested second.

response was far greater than a simple addition of the two independent effects.

Exclusion of Acetylcholine, Choline, and Histamine as Agents Causing Activity in Splenic Extracts

The reduction by atropine of the depressor response in the anaesthetized cat suggested the presence of choline or of its esters in the splenic extracts. Fresh spleen contains acetylcholine and choline in amounts of 5 to 30 μg./g. and 2 mg./g. respectively (Dale and Dudley, 1929; Chang and Gaddum, 1933).

Acetylcholine is well known to have a negative inotropic action on cardiac tissue, and when tested on papillary muscle only depressant effects were observed. Rather high concentrations, about 10^{-7} g./ml., were required to depress the contractile force by 1/3. This effect took about 1 min. to appear, and wore off in about 5 min. Fresh spleen can yield 9×10^{-5} g. acetylcholine from 3 g. of tissue, and a 9×10^{-5} g./ml. solution of acetylcholine added to a papillary muscle resulted in a decline in the contractile force to about half the control level.

It was unlikely that acetylcholine was present in the extracts, since the tissue was minced in air, which Dale and Dudley (1929) have shown to result in rapid enzymatic hydrolysis of acetylcholine. Thus only choline should be present in these extracts. *Extraction* (2) would yield a concentration of choline of 6×10^{-3} g./ml., assuming all the choline was extracted from 3 g. fresh tissue/ml. This dose of choline depressed papillary muscle.

Similarly, the histamine content of spleen is about 8 μg./g. of fresh tissue (Dale and Dudley, 1929; von Euler and Purkhold, 1951b). If complete extraction of histamine had occurred, the concentration of histamine would have been 2.4×10^{-3} g./ml. in the strongest samples of the least potent extract. Histamine in a concentration of 10^{-4} g./ml. caused only negative inotropic effects on papillary muscle. Lower concentrations, 10^{-5} g./ml. or less, were without effect.

The acetylcholine and histamine in samples of *Extraction* (2) were assayed on isolated guinea-pig intestine. Some extract was incubated at 37°, *p*H 11 for 1 hr. and then tested to see if catechol amines were present and inhibiting the stimulation. Doses of untreated and treated extract were equipotent on the guinea-pig gut. Constant submaximal responses of similar size were obtained with acetylcholine, histamine, and the splenic extract (Fig. 4). With small doses of atropine sulphate (5×10^{-10} to 1.5×10^{-9} g./ml. in the bath) there was progressive abolition of the response to acetylcholine, but the response to histamine was only slightly affected by higher doses. The response to splenic extract, whether incubated or not, was partially abolished, suggesting that only part of its stimulation was due to choline-like materials (Fig. 4). If all the action of splenic extract had been due to acetylcholine the concentration would have been 0.001 μg./g. of fresh tissue, or for choline, 1 μg./g. fresh tissue, allowing an activity ratio of acetylcholine to choline of 1,000 (Dale and Dudley, 1929).

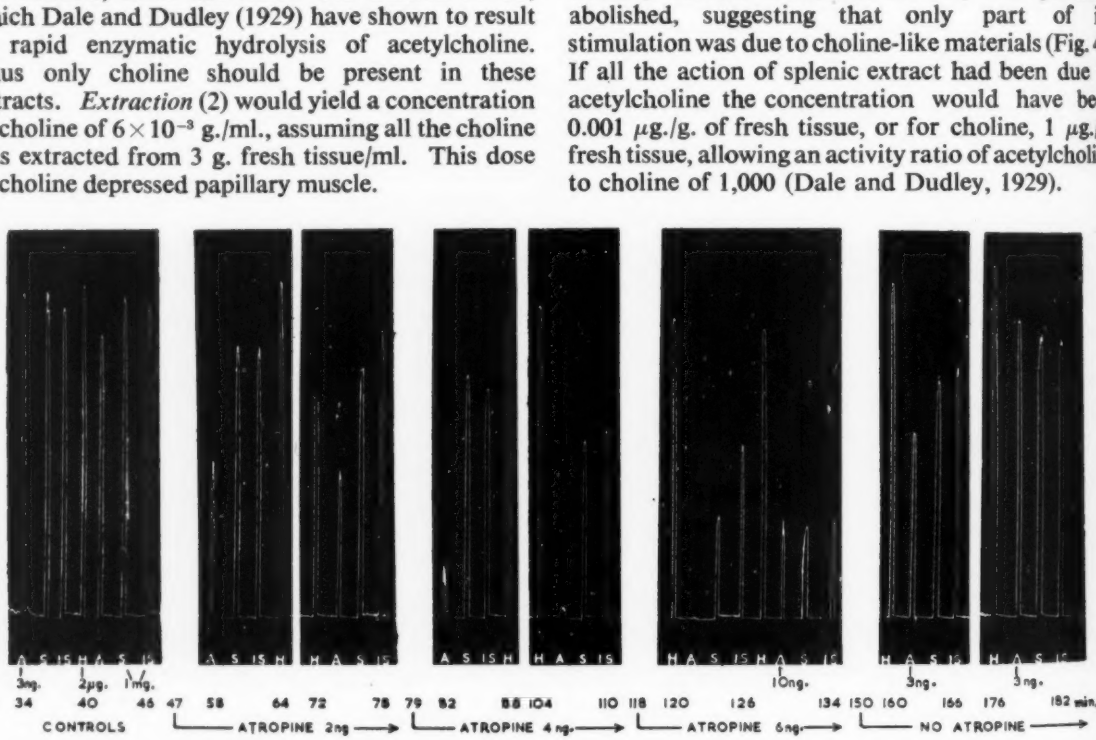

FIG. 4.—The effects of splenic extract [*Extraction* (2)], histamine and acetylcholine on the isolated guinea-pig intestine in a 4 ml. bath. ***A***, acetylcholine; ***H***, histamine; ***S***, splenic extract; ***IS***, splenic extract after incubation at *p*H 11.0 for 60 min. at 37° in the presence of O_2 and MnO_2. Atropine was present where indicated. All doses refer to the quantities added to the bath indicated in the left-hand panel, and are constant except where otherwise stated.

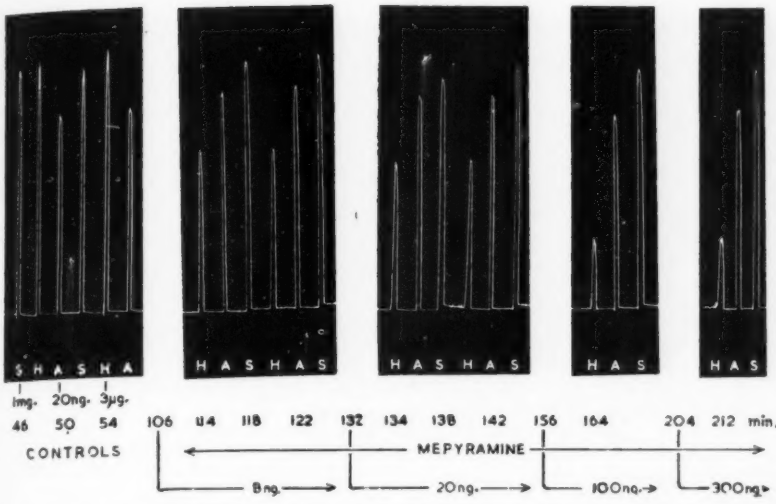

FIG. 5.—The action of splenic extract [*Extraction* (2)] upon isolated guinea-pig intestine in the presence of mepyramine maleate. All doses refer to quantities added to the bath. Abbreviations as in Fig. 4.

Similar experiments were performed using mepyramine maleate (2×10^{-9} g./ml. to 7.5×10^{-8} in the bath) as an antagonist. The response to histamine was gradually diminished, but the actions of acetylcholine and the splenic extract were not affected (Fig. 5). Thus very little of the smooth muscle stimulation by splenic extract could be attributed to histamine. However, if all the contraction following a dose of splenic extract had been due to histamine, the maximum amount present would have corresponded to 1 μg./g. of fresh spleen. Computation of the maximum histamine content from Fig. 4 gives a similar result. (An estimate from Fig. 5 of the maximum acetylcholine content of fresh spleen yields a seven-fold higher result, and since this was the highest value obtained in this series of experiments, it has been used in the work reported below.)

In experiments with *Extraction* (2) on papillary muscles, using a dose equivalent to 3 g. of fresh tissue/ml., the concentrations of histamine and choline would have been less than 3×10^{-6} g./ml. and 2.1×10^{-5} g./ml. respectively. As reported above, these concentrations were without effect on papillary muscle. It was concluded, therefore, that neither histamine, acetylcholine, nor choline were responsible for the inotropic activity of the splenic extract.

Figs. 4 and 5 show that about one-half of the stimulation of smooth muscle by the splenic extract could have been due to cholinergic materials of which atropine is an antagonist, and that very little could have been due to histamine.

Exclusion of 5-Hydroxytryptamine as an Agent Causing Activity in Splenic Extracts

5-Hydroxytryptamine is another smooth muscle stimulant known to occur in spleen which might have caused the residual activity. Since 5-hydroxytryptamine is soluble in acetone, it seemed probable that the residual activity on guinea-pig gut could be due to this substance.

Ox spleen contains up to 8 μg. of 5-hydroxytryptamine/g. of tissue (Erspamer, 1954). The 5-hydroxytryptamine in the extract was assayed on the isolated uterus of the virgin rat, a tissue insensitive to histamine, and about 1,000 times less sensitive to acetylcholine than to 5-hydroxytryptamine. Unlike guinea-pig intestine, the uterus of the virgin rat does not show tachyphylaxis to 5-hydroxytryptamine.

Constant submaximal responses to acetylcholine, 5-hydroxytryptamine, and the splenic extract were obtained (Fig. 6). In the presence of atropine (5×10^{-8} g./ml.) the responses to acetylcholine were blocked, but those to 5-hydroxytryptamine and splenic extract were unaffected. Lysergic acid diethylamide (3×10^{-9} g./ml.) antagonized the actions of 5-hydroxytryptamine and the splenic extract to similar extents, while the response to acetylcholine was hardly affected.

The doses (1 mg.) of *Extraction* (2) used in the rat uterus experiments would have contained 0.02 mg. of acetylcholine/mg. of extract, if this drug had been responsible for their activity. However this dose of acetylcholine is sub-threshold on the rat uterus. In view of the similarity in behaviour of the splenic extract and 5-hydroxytryptamine, it is not unreasonable to assume that all of the oxytocic activity of the extract is due to 5-hydroxytryptamine; the maximum quantity of 5-hydroxytryptamine in the extract being 0.02 μg./g. of fresh tissue.

When tested on papillary muscle, 5-hydroxytryptamine had a positive inotropic action. No action was seen at concentrations of 10^{-6} g./ml. or less. At a concentration of 5×10^{-5} g./ml., the force of contraction was increased by about 50% and reached a maximum in 5 to 6 min. Higher concentrations (10^{-5} and 10^{-4} g./ml.) doubled and quadrupled respectively the force of contraction, and

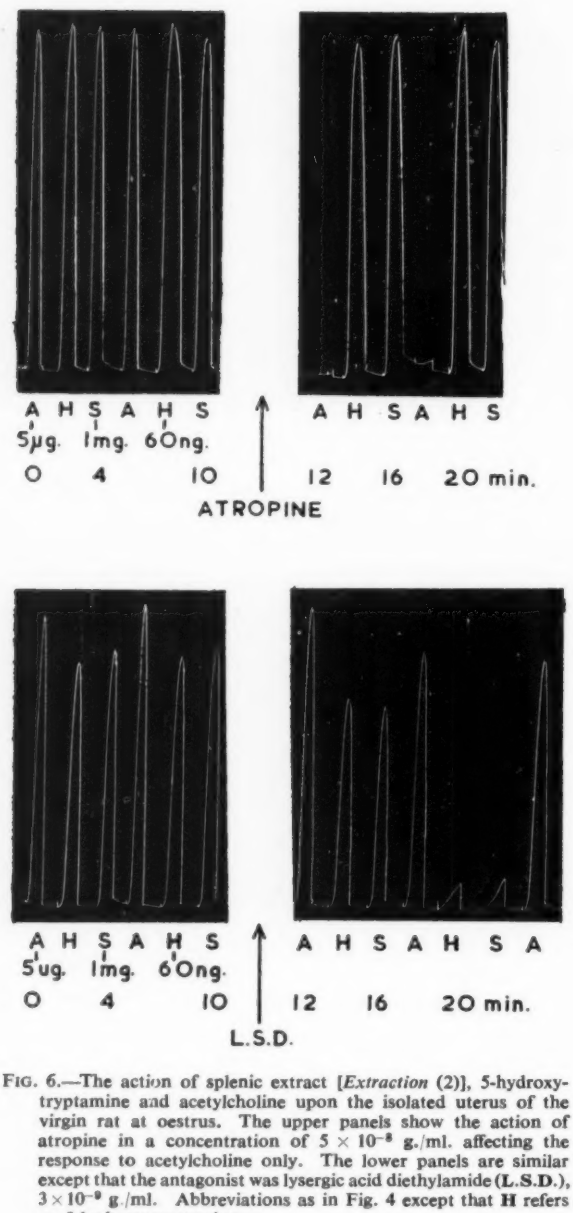

FIG. 6.—The action of splenic extract [*Extraction* (2)], 5-hydroxytryptamine and acetylcholine upon the isolated uterus of the virgin rat at oestrus. The upper panels show the action of atropine in a concentration of 5×10^{-8} g./ml. affecting the response to acetylcholine only. The lower panels are similar except that the antagonist was lysergic acid diethylamide (L.S.D.), 3×10^{-9} g./ml. Abbreviations as in Fig. 4 except that H refers to 5-hydroxytryptamine.

with both strengths the response reached a maximum in about 5 min. The action lasted well over 1 hr. when the concentration was 10^{-5} g./ml.

When 5-hydroxytryptamine was dissolved in modification (c) of Krebs-Hensleit solution at a concentration of 10^{-5} g./ml. and subjected to the alkaline incubation in the presence of MnO_2 and O_2 at *p*H 11, 37° for 1 hr., all the positive inotropic action disappeared. As reported earlier the splenic extract retained its activity under these conditions.

If all the 5-hydroxytryptamine in the sample of spleen had been extracted into the acetone, the concentration in the papillary muscle bath would have been 2.4×10^{-5} g./ml. in experiments using extracts from 3 g. fresh tissue/ml. This is very close to the threshold dose of 5-hydroxytryptamine. Using the maximum value for 5-hydroxytryptamine actually found in the extract when assayed on the rat uterus, the concentration of 5-hydroxytryptamine would only have been 6×10^{-8} g./ml., which is considerably below the threshold for papillary muscle. Thus all our evidence is against 5-hydroxytryptamine being responsible for the positive inotropic action of splenic extract on isolated papillary muscle of the cat.

The maximum quantities of catecholamines, histamine, choline, and 5-hydroxytryptamine found are given in Table II. In fact, the actual quantities of most of these substances were considerably less than the values quoted. Table II also contains the quantities of these materials which would have been present in a papillary muscle experiment using the equivalent of 3 g. of fresh spleen/ml. For comparison, the mean quantities reported elsewhere of these substances are included.

TABLE II

MAXIMUM QUANTITIES OF SOME KNOWN MATERIALS FOUND IN *EXTRACTION* (2)

The quantities (in µg.) which would have been present in the 20 ml. bath in a papillary muscle experiment using the equivalent of 3 g. of fresh tissue/ml. are also given in col. (3). For comparison, the mean values obtained from the literature are quoted with the respective source. References in col. (5): 1, von Euler and Purkhold (1951a); von Euler (1950); von Euler (1954); 2, Dale and Dudley (1929); 3, von Euler and Purkhold (1951b); and 4, Erspamer (1954).

Substance	Acetone Extract of Spleen		Values Reported by Other Workers	
	(µg./g. Fresh Tissue)	Quantity Bath	µg./g. Fresh Tissue	References
(1)	(2)	(3)	(4)	(5)
Adrenaline ..	None found	None	0·07	1
Noradrenaline	"	"	4·0	1
Choline ..	7·0	420	2,000	2
Histamine ..	1·0	60	8	2, 3
5-Hydroxytryptamine	0·02	1·2	8	4

To be quite certain that these known materials did not interact to cause a positive inotropic action on papillary muscle, the maximum quantities which could have been present were combined together in a " cocktail " and assayed. No inotropic activity was observed, and it was concluded that the cardiotonic factor in spleen was neither adrenaline, noradrenaline, choline, acetylcholine, histamine, 5-hydroxytryptamine, nor a combination of these.

Discussion

From Table II it is apparent that the spleen contains a number of biologically active materials of considerable potency. The neurohormones acetylcholine, adrenaline, and noradrenaline are usually considered to be present in an amount proportional to the degree of innervation of the tissue. Since the spleen is innervated both by vagal nerve fibres and by sympathetic postganglionic nerve fibres from the coeliac ganglion, these neurotransmitters should be present in reasonably large quantities. The presence of histamine in this tissue is not so easily explained; there is about the same quantity as of acetylcholine. Finally, Erspamer (1954) has explained the relatively large concentrations of 5-hydroxytryptamine as originating from the serum and in particular from degenerating platelets.

To obtain these materials from the spleen various authors have used extraction methods designed to yield the maximum quantity of each of the substances. For acetylcholine, choline, and histamine the solvent employed was cold alcohol and, to prevent rapid destruction of acetylcholine, the tissue had to be minced in the solvent. To extract the total sympathomimetic neurohormones from spleen either acidified ethanol or trichloracetic acid was used (von Euler, 1950). 5-Hydroxytryptamine was prepared from splenic tissue by the use of an acetone extract (Erspamer, 1940a, 1940b). In the extracts used in this study it is not surprising that lesser quantities of known materials than those reported in the literature were found. Since the spleen extract was prepared without the special precautions necessary to preserve acetylcholine, the only cholinergic activity present in the acetone fraction was most probably due to choline. This supposition is supported by the fact that incubation of the extract under alkaline conditions did not diminish the stimulant action on the isolated guinea-pig ileum.

The stimulation of smooth muscle by the extracts could have been due to choline or its esters since the action was partially blocked by atropine. The residual stimulation was not due to histamine since there was no diminution of response in the presence of mepyramine. When tested for 5-hydroxytryptamine on the isolated rat uterus the splenic extract was found to contain a small quantity which would account for the residual activity on guinea-pig ileum in the presence of atropine. Thus it is most likely that the smooth muscle stimulation by the splenic extract can be explained in terms of its content of choline and 5-hydroxytryptamine. It is strange that the yield of 5-hydroxytryptamine in the extract should be so low compared with the concentrations found by others in ox spleen. The only explanations which can be offered are either that the extraction of 5-hydroxytryptamine was incomplete, or that it could have been eliminated during the removal of fat soluble materials from the crude acetone fraction.

The fact remains that a cardiotonic principle is present in an acetone extract of spleen. This principle cannot be histamine, choline, or choline esters since these were found to have only a depressant action on the force of contraction of isolated papillary muscle, even when given in the maximum concentrations at which they might occur (as shown by the smooth muscle experiments). The active material was neither adrenaline nor noradrenaline since it was not destroyed on alkaline incubation in the presence of MnO_2 and O_2, whereas all the noradrenaline in an equivalent quantity of fresh spleen was destroyed by this procedure. Further evidence that noradrenaline was not responsible is that added noradrenaline was destroyed by incubation at *p*H 11, showing that the splenic extract did not contain a substance protecting noradrenaline from destruction.

5-Hydroxytryptamine is interesting since concentrations of 5×10^{-6} g./ml. are sufficient to cause a regular increase of about 50% in the force of contraction of isolated papillary muscle. Green and Nahum (1957) found the same order of activity although Loubatières, Sassine, and Mauche (1955) reported that concentrations up to 10^{-4} g./ml. had no inotropic effect. When 5-hydroxytryptamine (10^{-5} g./ml.) was incubated with MnO_2 and O_2 at *p*H 11.0, the inotropic action was destroyed. Quantities of 5-hydroxytryptamine as small as those in our extracts were without effect on papillary muscle and consequently the cardiotropic principle cannot be due to 5-hydroxytryptamine. Erspamer (1942) has shown that 5-hydroxytryptamine is partly inactive when first extracted, but that it may be fully activated either by heating for 10 min. at 100° at *p*H 6 to 8 or by incubating in buffer solution at 40° and *p*H 9 to 11 for 1 hr. or longer. A similar procedure did not increase the activity of the splenic extract but destroyed the action of pure 5-hydroxytryptamine on the heart. It therefore seems safe to conclude that low apparent concentrations of 5-hydroxytryptamine in the extracts (assayed on the rat uterus) were not increased by alkaline incubation, and even if activation had occurred initially it would have been abolished by continuing the incubation for 1 hr.

The final proof that histamine, acetylcholine, choline, and 5-hydroxytryptamine were not responsible for the activity of the acetone extract was

that, when the maximum concentrations at which the drugs could occur in *Extraction* (2) were combined and tested on papillary muscle, no inotropic effect was observed.

As to the nature of the cardiotonic principle in spleen we have the following information: it is soluble in acetone and water, is not destroyed by incubation at alkaline *p*H in the presence of MnO_2 and O_2 and it is ultrafiltrable. A material soluble in acetone has been extracted from various bodily tissues and identified as palmitoyl lysolecithin (Titus, Weiss, and Hajdu, 1956; Hajdu, Weiss, and Titus, 1957).

These authors did not include the spleen among the organs surveyed for palmitoyl lysolecithin. Their techniques for estimation of cardio-activity included measuring the abolition of the staircase phenomenon in the isolated frog heart and determining the increase in systolic tension of isolated right ventricular muscle of the squab. It is doubtful whether information derived from these tests correlates with effects upon the mammalian heart since, in experiments performed in this laboratory, synthetic palmitoyl lysolecithin (in concentrations up to 50 μg./ml.) caused no increase in the tension developed by isolated papillary muscle.

It remains to be determined whether the substance in the spleen is related to the "*hypoxie-lienen*" of Bücherl and Rein (1949). Rein (1942) suggested that a digitalis-like material was present in the body and it is possible that a steroid might appear in an acetone extract. Whether or not a steroid would be stable on alkaline incubation would depend on the peripheral chemical groupings surrounding the nucleus.

The spleen-liver reaction of Rein *et al.* (1949) involves more economical and efficient function in a heart failing from hypoxia. Noradrenaline (and adrenaline) cannot account for the spleen-liver reaction since the effects of noradrenaline are too transient (Meesmann and Schmier, 1955). Meesmann and Schmier (1956) have shown that the spleen-liver reaction could not be due to a variety of known substances including 5-hydroxytryptamine, adenyl compounds, and commercial spleen and liver extracts.

Further work is in progress to identify the cardiotonic material in these extracts of freeze dried spleen.

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THE ACTION OF NITROPHENOLS ON THE PULMONARY VENTILATION OF RATS

BY

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(RECEIVED MAY 25, 1959)

The ventilatory effects of the three mononitrophenols and six dinitrophenols have been examined in anaesthetized rats. The minute volume of ventilation increased in all the test groups, the increase reaching the 99% significance level with seven compounds ($P<0.01$), the 95% level with 3-nitrophenol ($P<0.05$), and the 90% level with 3,5-dinitrophenol ($P<0.10$). The effects of 4-nitrophenol, 3,4-dinitrophenol, 3-nitrophenol, and 2,5-dinitrophenol, in increasing carbon dioxide output relative to oxygen consumption, are not explicable on the basis of simple hyperventilation, and are attributed to a metabolic effect at the cellular level. The potency of the nitrophenols in stimulating respiration is related to their structure, nitro groups being most effective in the *ortho* position and least effective in the *meta* position, and 2,4-dinitrophenol being the most powerful respiratory stimulant of the group. 2,6-Dinitrophenol does not conform to this generalization; one unique feature of its structure is indicated, as a possible explanation for the discrepancy. The gradation of potency of the nitrophenols (except 2,6-dinitrophenol) parallels the gradation of acidic properties in the group; the more strongly acid compounds stimulate respiration more powerfully. This is not a direct effect on blood *p*H, since the compounds were administered in neutral or slightly alkaline solution. Methaemoglobin formation was found to occur with 2,5-dinitrophenol, and to a smaller inconstant extent with three other compounds. Further work is suggested, to explore whether peripheral-acting ventilatory stimulation by 2,4-dinitrophenol is necessarily associated with peripheral metabolic enhancement, or whether the two effects can be dissociated.

The three mononitrophenols and six dinitrophenols have been compared by Cameron (1958) in respect of their effects on oxygen consumption of the rat. She found that 4-nitrophenol and 3,4-dinitrophenol increase carbon dioxide output without affecting oxygen uptake, and that 3-nitrophenol and 2,5-dinitrophenol depress oxygen consumption without affecting carbon dioxide output. While such relative stimulation of carbon dioxide output could be due to deviation from normal metabolic pathways at the cellular level, it might also be due to hyperventilation, and would in any case be modified by a change in the respiratory minute volume. To decide between these two possibilities, a study has been undertaken of the effects on ventilatory rate and depth of all nine of the compounds, including 2,4-dinitrophenol, the only true metabolic stimulant in the group.

METHOD

A pneumotachograph (Lilly, 1950) was constructed using the manometer of Greer (1958) and a low-inertia integrating motor. This was set up to record ventilatory volume over 53 sec. periods and respiratory rate over 30 sec. periods, and to allow monitoring on an oscilloscope of the respiratory airflow pattern (Silverman and Whittenberger, 1950).

Twelve anaesthetized tracheotomized rats could be studied at a time, allowing the ventilation of each to be measured four times/hr.

Wistar albino rats were used, in the age range 3 to 5 months. The mean weights were 270 g. (males) and 190 g. (females) with coefficient of variation 15%.

For anaesthesia, 16% ethanol by volume was given by stomach tube at an initial rate of 4.5 ml./100 g., and supplemented as necessary until preparations were obtained which would remain satisfactorily anaesthetized for the 2½ hr. period of the experiment.

The nine compounds were made up with sodium hydroxide in neutral or slightly alkaline solution

(Table III) and were administered in doses up to the maximum tolerable level as determined by Cameron (1958). A result was rejected if the three values to be averaged differed by more than 50%, if the animal died or needed more anaesthetic during the experimental period, or if respiration as monitored by the oscilloscope was gasping in character, or showed persistent irremediable airway obstruction.

Experimental Design.—After control observations over 1 hr., each animal received an intraperitoneal injection of normal saline or of a test compound; respiratory measurements were then continued for 1.5 hr. The first three observations of minute volume in the test period were averaged, and compared with the average of the last three control period observations, results being expressed as the ratio, average minute volume of respiration in test period/average minute volume of respiration in control period. A histogram of the frequency distribution of this ratio for 66 control animals showed positive skewness so the logarithms of the ratios were used for a comparison of each test group (9 to 21 animals) with the control group, using Cochran's modification of Student's t-test (Snedecor, 1956).

Results

The effects on respiratory minute volume are shown in Table I. Ventilation in the control period averaged 125 ml./min., with a mean rate of 78 respirations in each 30 sec. period.

TABLE I

EFFECT OF NITROPHENOLS ON MINUTE VOLUME OF RESPIRATION OF WISTAR ALBINO RATS ANAESTHETIZED WITH ETHANOL

Compound	Dose Range (mg.)	No. of Rats	% Increase in Respiration Compared with Control Period. Mean with S.E.		t	P
Control group ..	—	66	3·3	1·6	—	—
2,4-dinitrophenol	1–4	9	60	16	4·20	< 0·01
2,3-dinitrophenol	10–15	10	47	6	8·04	< 0·001
2,5-dinitrophenol	7–25	21	32	8	3·96	< 0·001
2-nitrophenol ..	60–120	16	27	7	3·66	< 0·01
4-nitrophenol ..	7–12	19	24	4	4·52	< 0·001
3,4-dinitrophenol	2–12	21	23	7	3·06	< 0·01
2,6-dinitrophenol	1–6·5	17	21	5	3·24	< 0·01
3-nitrophenol ..	20–45	17	15	5	2·15	< 0·05
3,5-dinitrophenol	4–7	16	11	4	1·98	< 0·10

In the test period, ventilation increased with all the compounds. The increase was significant at the 99% confidence level in seven instances ($P<0.01$), at the 95% level in one and at the 90% level in one. The effects on respiratory rate and tidal volumes are set out in Table II; in seven instances both rate and depth of respiration were increased, but in two the increased minute volume was produced entirely by tachypnoea.

Blood samples were examined for methaemoglobinaemia, using a reversion spectroscope.

TABLE II

EFFECTS OF NITROPHENOLS ON RAT RESPIRATION IN TEST PERIOD COMPARED WITH CONTROL PERIOD

Compound	Increase in Rate (%)	Increase in Tidal Volume (%)
2,4-dinitrophenol	16	37
2,3-dinitrophenol	12	31
2,5-dinitrophenol	9	21
2-nitrophenol	31	−5
4-nitrophenol	2	21
3,4-dinitrophenol	11	10
2,6-dinitrophenol	5	15
3-nitrophenol	24	−7
3,5-dinitrophenol	1·5	9
Control group	2	1

Substantial methaemoglobin formation was found with 2,5-dinitrophenol, and smaller inconstant amounts were formed with 2-nitrophenol, 2,3-dinitrophenol and 3,4-dinitrophenol.

Discussion

The wire screen pneumotachograph was well suited for studies in the rat, since there were no moving parts or valves to impede respiration, and the resistance of the screen was very low (about 1 mm. of water). Continuous monitoring on an oscilloscope of the air-flow pattern (pneumotachogram) provided a sensitive check on the character of respiration, and rapidly detected airway obstruction.

Methaemoglobinaemia (Bodansky, 1951) is associated with normal arterial oxygen tension, and does not produce respiratory distress unless the amount of normal haemoglobin is markedly reduced. The experimental results for 2,5-dinitrophenol should be treated with reserve, but the other results are not thought to be materially affected by methaemoglobin formation.

The present results differ completely from the visual observations of Cameron (1958) on the apparent depth and rate of respiration in her experiments. This illustrates the necessity for quantitative measurements of ventilatory volume in work of this type.

The dose ranges used for the different compounds are equivalent in that they represent approximately equal fractions of the lethal dose. Inspection of Table I shows a relation between structure and degree of respiratory stimulation. A nitro group in the *ortho* position is more potent than one in the *para* position; and a *meta* substituted group is least effective. 2,6-Dinitrophenol fails to conform to this structure function pattern; it may be relevant that this compound is unique in the series in having side chains on both sides of the hydroxyl group. A similar gradation

from *ortho* through *para* to *meta* exists in respect of the acidic properties of the compounds, as shown in Table III. The compounds were administered in neutral or slightly alkaline solution (*p*H 6 to 9, Table III) so the increases in ventilation were not caused by hydrogen ion administration.

TABLE III

COMPARISON OF EFFECTS OF NITROPHENOLS ON RAT RESPIRATION WITH THEIR ACIDIC PROPERTIES

Concentration and *p*H of drug solutions administered are given. *p*KA values at 25° obtained from Pauling (1940).

Compound	Increase in Respiratory Volume (%)	*p*KA	Conc.	*p*H
2,4-dinitrophenol ..	60	4·00	1%	7·1
2,3-dinitrophenol ..	47	4·92	3%	7·0
2,5-dinitrophenol ..	32	5·15	1%	7·7
2-nitrophenol ..	27	7·12	3%	..
4-nitrophenol ..	24	7·02	1%	8·1
3,4-dinitrophenol ..	23	5·43	2%	7·4
2,6-dinitrophenol ..	21	3·70	1%	6·2
3-nitrophenol ..	15	8·00	1%	8·4
3,5-dinitrophenol ..	11	6·68	2%	7·6

If the excess liberation of carbon dioxide were caused by hyperventilation, it would affect all the compounds in proportion to their effect on ventilation. If this were the only effect of the group on carbon dioxide metabolism, increased carbon dioxide production would vary in strict proportion to the effect on ventilation. The results do not show such a correlation, and prove that hyperventilation cannot be the sole cause of the effect on carbon dioxide production. The complete absence of any correlation suggests that hyperventilation is not even an important factor, and that the carbon dioxide effect is due to an action on cell respiration.

There is evidence that 2,4-dinitrophenol produces at least some of its hyperventilatory effect by stimulation of peripheral receptors, probably in muscle (Ramsay, 1955, 1959). It is tempting to suggest that the other nitrophenols act in the same way. Since these compounds do not increase oxygen uptake, it would then follow that peripheral metabolic enhancement was not an essential step in the peripheral ventilatory stimulus. The present results cannot settle the question, which needs further experiment.

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THE PHARMACOLOGY OF A MOLLUSCAN SMOOTH MUSCLE

BY

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The effects of a number of pharmacologically active substances on contraction and on membrane polarization of the anterior byssal retractor muscle of *Mytilus edulis*, L., have been studied. Tetramethylammonium bromide, trimethyl(4-oxopentyl)ammonium chloride and nicotine, like acetylcholine, produced depolarization and sustained contraction. Nicotine, on repeated application, lost acetylcholine-like activity and effectively blocked acetylcholine. In order of decreasing potency, methanthelinium, tubocurarine, benzoquinonium, tetraethylammonium, atropine, pentamethonium, and decamethonium blocked acetylcholine action. These agents did not show initial acetylcholine-like action and did not relax sustained contractions. Adrenaline, noradrenaline, tyramine, dibenamine, phentolamine, and lysergic acid diethylamide relaxed sustained contractions without reducing initial depolarization and tension development in response to acetylcholine or electrical stimuli. Adrenaline and noradrenaline often caused depolarization and contraction when first applied, and displayed relaxing action on subsequent application.

By varying duration and frequency of stimulating electrical pulses, either a brief or a prolonged contraction may be evoked in the anterior byssal retractor muscles of *Mytilus edulis*, L. The prolonged contraction can be abruptly relaxed by repetitive electrical stimuli. These effects can be imitated by application of pharmacological agents, outstanding among these being acetylcholine and 5-hydroxytryptamine, which are naturally present in Mytilus muscle (Twarog, 1954) and ganglia (Welsh, personal communication). Acetylcholine produced depolarization and prolonged contraction. 5-Hydroxytryptamine promptly relaxed prolonged contractions. Acetylcholine and electrical stimuli, in the presence of 5-hydroxytryptamine, produced large, brief contractions and, often, rhythmic contractile activity.

Although the actions of acetylcholine, 5-hydroxytryptamine and adrenaline have been described in some detail (Twarog, 1954), the action of other substances on the anterior byssal retractor muscles has been only briefly reported (Twarog, 1952). The mechanisms of prolonged contraction and of relaxation are intrinsically interesting and the wide variety of agents affecting these phenomena is not generally known. It is the purpose of this note to make available some further, although incomplete, pharmacological results.

METHODS

In the study of acetylcholine-like, of acetylcholine-blocking and of relaxing agents, the muscle was mounted in a partitioned chamber to permit simultaneous recording of changes in demarcation potential and mechanical responses (Twarog, 1954).

In experiments comparing the relative acetylcholine-blocking efficiency of various agents, the muscles were mounted in upright chambers which were perfused with aerated sea water. Contractions were recorded on a moving drum by an isotonic lever weighted with 15 g. The height of contraction was measured before and after soaking for 2 min. in solutions of blocking agents, and % block calculated from the ratio of the magnitude of the response to acetylcholine after treatment to that before treatment.

RESULTS

Acetylcholine-like Agents.—Tetramethylammonium bromide, in concentrations above 5×10^{-6} M and trimethyl(4-oxopentyl)ammonium chloride (4-ketoamyltrimethylammonium chloride), an active stable analogue of acetylcholine (Welsh and Taub, 1950, 1951; Ing, 1949) in concentrations above 10^{-6} M, induced depolarization and a prolonged contraction. Characteristically, maximum depolarization was attained later than with acetylcholine. Depolarization followed the approximately exponential curve obtained after application of acetylcholine to a preparation treated with eserine.

Nicotine, below 10^{-4} M, potentiated the action of acetylcholine. Above 10^{-4} M, it evoked depolarization and prolonged contraction. However, tachyphylaxis was pronounced. With repeated applications of or continued soaking in nicotine, the

acetylcholine-like activity ceased. Loss of activity was associated with an acetylcholine-blocking action.

Acetylcholine-blocking Agents.—Nicotine, after acetylcholine-like activity had been lost, blocked acetylcholine very effectively. The blocking action could be reversed only with difficulty. Decamethonium, in concentrations lower than 5×10^{-4} M, potentiated acetylcholine action, suggesting some acetylcholine-like activity, weaker, however, than that of nicotine. Other substances have been studied which, in concentrations between 10^{-5} M and 5×10^{-4} M, blocked without displaying acetylcholine-like action. These are listed in Table I, in order of decreasing potency.

TABLE I

BLOCK OF THE ACTION OF 4×10^{-6} M ACETYLCHOLINE AT VARIOUS MOLARITIES OF SOME BLOCKING AGENT

An asterisk indicates potentiation of acetylcholine action.

Blocking Agent	% Block		
	10^{-5} M	10^{-4} M	5×10^{-4} M
Methanthelinium bromide ..	80	97	—
Tubocurarine	56	91	—
Benzoquinonium chloride ..	30	99	—
Tetraethylammonium	30	78	—
Atropine	20	98	—
Pentamethonium bromide ..	16	43	100
Decamethonium ,, ..	*	*	100

The acetylcholine-blocking drugs reduced depolarization by acetylcholine and tension development was also reduced to approximately the same degree as depolarization. The blocking action was reversible. The general form of contraction in response to acetylcholine was not altered, but tension was diminished and finally abolished entirely. These agents, applied during the phase of sustained tension after acetylcholine or electrical stimulation, did not relax the muscle.

Relaxing Agents.—Noradrenaline, in concentrations above 10^{-7} M, induced depolarization and prolonged contraction, summating with acetylcholine. When noradrenaline was not washed off, or was applied repeatedly in concentrations of 10^{-4} M or more, relaxation of tension ensued. No marked change in membrane polarization accompanied relaxation. With a maintained concentration of 5×10^{-4} M noradrenaline (in contrast to 10^{-8} M or 10^{-7} M 5-hydroxytryptamine), larger, but briefer, contractions followed acetylcholine or electrical stimulation.

The relaxing action of adrenaline, previously reported (Twarog, 1954), was more striking than that of noradrenaline. The concentrations at which these agents acted differed by about ten fold, adrenaline being the more potent.

Tyramine did not stimulate a resting muscle. In 10^{-4} M to 10^{-3} M tyramine, the contractile response to acetylcholine or electrical stimulation was increased while the sustained phase of contraction was abolished.

The anti-adrenaline agents dibenamine and phentolamine in concentrations of 10^{-5} M potentiated the contractile response to acetylcholine and electrical stimulation and relaxed prolonged tension (Fig. 1) as did higher concentrations of adrenaline, noradrenaline, and tyramine. The effect of these agents developed rather slowly and that of dibenamine appeared to be irreversible.

Certain lysergic acid derivatives also showed a relaxing action. This group included both anti-adrenaline agents and substances which affected or imitated the action of 5-hydroxytryptamine. Ergotamine and dihydroergotamine displayed no relaxing activity even in saturated solutions (about 10^{-4} M in sea water). Ergometrine, at 10^{-4} M or greater concentrations, effectively relaxed; it showed no excitatory action. Lysergic acid diethylamide (10^{-5} M) relaxed, again without producing initial depolarization or contraction.

DISCUSSION

Trimethyl(4-oxopentyl)ammonium ions were equal in potency to acetylcholine before but were about ten times less effective than acetylcholine applied to a muscle after treatment with eserine. Tetramethylammonium ions were about five times less potent than trimethyl(4-oxopentyl)ammonium ions. The curves of depolarization and repolarization closely resembled those for acetylcholine after eserine. Neither tetramethylammonium nor trimethyl(4-oxopentyl)ammonium ion would be inactivated by cholinesterase. This supports a previous conclusion that both potency and rate of depolarization and repolarization with acetylcholine are limited by cholinesterase activity in the fresh muscle.

The relative potencies of acetylcholine-blocking agents indicated that the anterior byssus retractor could not be classified as nicotinic or muscarinic, but was susceptible to agents blocking acetylcholine-like action at the neuromuscular junction and at autonomic ganglia as well as on smooth muscle of vertebrates. It resembled in some respects the heart of *Venus mercenaria* (Welsh and Taub, 1953; Luduena and Brown, 1952) except that benzoquinonium was not so outstandingly active. Significantly, these agents reduced the initial action of applied acetylcholine but, unlike the relaxing agents, did not affect tension maintenance. This could indicate that tension is maintained through a

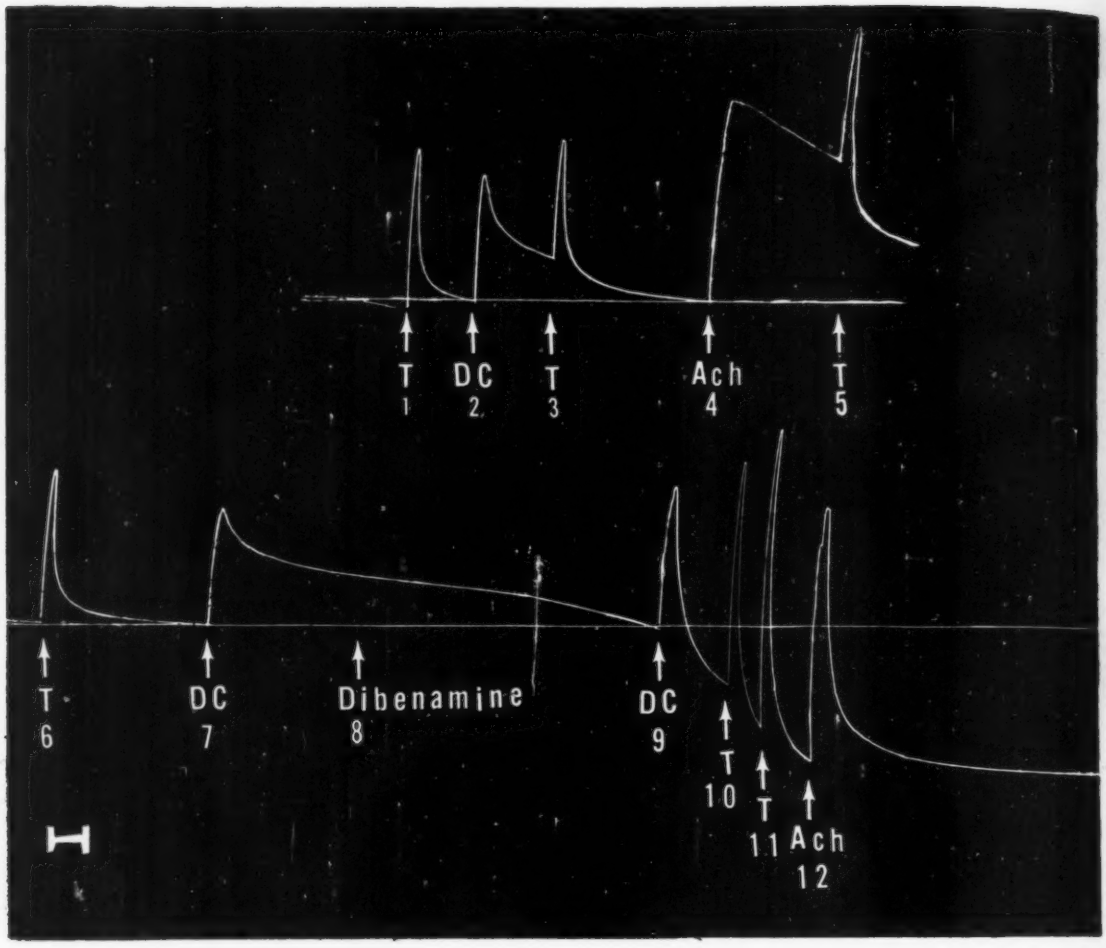

FIG. 1.—The effects of dibenamine on the responses of the anterior byssal retractor muscle of *Mytilus* to repeated, brief electrical pulses (T), single, long pulses (DC) and to acetylcholine (Ach). (1) 15 v. anodal, 20 msec., 10/sec. for 5 sec. (2) 3 v. cathodal, 5 sec. (3) 20 v. anodal, 20 msec., 10/sec. for 2.5 sec. (4) Ach 5×10^{-6} M for 10 sec. (5) 20 v. anodal, 20 msec., 10/sec. for 2.5 sec. (6) 5 v. anodal, 20 msec., 10/sec. for 3.5 sec. (7) 3 v. cathodal, 10 sec. (8) Dibenamine 10^{-4} M. (9) 3 v. cathodal, 10 sec. (10) 15 v. cathodal, 20 msec., 10/sec. for 2.5 sec. (11) 15 v. anodal, 20 msec., 10/sec. for 2.5 sec. (12) Ach 5×10^{-6} M for 15 sec. Time, 1 min.

non-cholinergic mechanism or is perhaps maintained without continued excitation (Twarog, 1958; Johnson and Philpott, 1959). Another interpretation is that acetylcholine released at an intramuscular site might not be as susceptible to block as applied acetylcholine. Evidence against this is the observation that methanthelinium and benzoquinonium block neural excitation of the byssal retractor (Twarog, unpublished observation). However, this blocking action required concentrations five to ten times greater than those necessary for full block of applied acetylcholine.

Relaxation of pre-existing tension without block of tension development has been observed with 5-hydroxytryptamine and related agents in the smooth muscle of the gastropod raduiar apparatus (Hill, 1958; Fänge and Mattisson, 1958) as well as in the dorsal muscle of the leech (Poloni, 1955). Adrenaline relaxes the gastropod radula muscle (Hill, 1958).

Hoyle and Lowy (1956) observed relaxation of the byssus retractor with lysergic acid diethylamide, and this observation was confirmed. Here, as in the Venus heart (Welsh and McCoy, 1957) and in many vertebrate smooth muscles, lysergic acid diethylamide mimicked rather than blocked 5-hydroxytryptamine.

Of the ergot derivatives, ergotamine and dihydroergotamine, the most active anti-adrenaline agents, did not display a relaxing action, while ergometrine, a very poor anti-adrenaline agent and the most active uterine-stimulating agent, relaxed. However,

dibenamine and phentolamine relaxed very effectively.

The reversal of adrenaline and noradrenaline actions with repeated dosage at moderately high concentrations suggests a mechanism of block by the building-up of a local excess (Burn and Robinson, 1951). Relaxation might then be due to such a block. To some extent the action of anti-adrenaline agents lends support, but the relative activity of the ergots weakens the blocking hypothesis. Further, the connexion between the high-concentration effect of adrenergic agents and the actions of 5-hydroxytryptamine remains obscure. The phenomenon merits further attention, particularly in view of the interest which attaches to the distribution of noradrenaline, adrenaline, and 5-hydroxytryptamine in vertebrate nervous centres and the action of agents such as reserpine in relation to the release of catechol amines and of 5-hydroxytryptamine from storage sites in nervous tissue.

The major portion of this research was conducted while the author was a Predoctoral Fellow of the Atomic Energy Commission in the Biological Sciences of the National Research Council. The work was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U.S. Public Health Service, to Professor J. H. Welsh at the Biological Laboratories, Harvard University.

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THE EFFECT OF NITROFURAZONE ON *TRYPANOSOMA RHODESIENSE* INFECTIONS IN MICE

BY

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From the East African Trypanosomiasis Research Organization, Tororo, Uganda

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Mice infected with a freshly isolated strain of *Trypanosoma rhodesiense* were not cured by ten daily intraperitoneal doses of 50, 100, 200, or 400 mg./kg. of nitrofurazone (5-nitrofurfuraldehyde semicarbazone). At the highest dosage used, the drug was lethal to some of the mice.

Nitrofurazone (5-nitrofurfuraldehyde semicarbazone; Furacin) was found to cure mice (Dodd, 1946; Giarman, 1951) and rats (Dodd, 1946) infected with *Trypanosoma equiperdum*. Dodd (1946) found that 150 mg./kg. cured 73.3% of the mice, while Giarman (1951) estimated the curative dose for 50% of the mice (CD50) to be 247.6 mg./kg. when given in a single subcutaneous injection and 263.7 mg./kg. orally. Packchanian (1955) showed that total quantities of 450 and 600 mg./kg. orally in nine or twelve daily doses, respectively, cured all mice infected with *T. gambiense*. Evens, Niemegeers, and Packchanian (1957a) found that 16 out of 32 guinea-pigs infected with various strains of *T. gambiense* were cured by single intramuscular doses of from 50 to 150 mg./kg. of nitrofurazone. However, only 3 out of 30 guinea-pigs infected with strains of *T. rhodesiense* were cured by similar doses. Evens, Niemegeers, and Packchanian (1957b) demonstrated that nitrofurazone was of some value in treating human cases of *T. gambiense* infection. The compound did not appear to cure *T. cruzi* infections (Packchanian, 1957).

The lethal dose for 50% of the animals treated (LD50) has been variously estimated as 545 mg./kg. (Dodd, 1946) and 735.8 mg./kg. (Giarman, 1951) orally to mice, 753.0 mg./kg. (Giarman, 1951) subcutaneously to mice, and about 450 mg./kg. (Evens *et al.*, 1957a) orally to rats.

In view of the above work it seemed worth while to determine the effect of nitrofurazone on a recently isolated strain of *T. rhodesiense*.

MATERIALS AND METHODS

Strain.—Strain No. SS 284 was isolated in albino rats inoculated with the blood of a Nyala fisherman believed to have become infected on Sigulu Island in the north-east corner of Lake Victoria, East Africa. The patient had been ill for four months before the isolation of the strain. The strain caused an acute infection in albino rats and was diagnosed on these and clinical grounds as *T. rhodesiense* Stephens and Fantham.

Animals.—Male albino mice (average weight 24 g.) of the closed colony maintained by the East African Trypanosomiasis Research Organization at Tororo, Uganda, were used.

Drug.—Nitrofurazone (5-nitrofurfuraldehyde semicarbazone; Furacin), kindly supplied by Messrs. Smith, Kline, and French, was prepared for inoculation by grinding 0.75 g. of drug with 0.25 g. of gum acacia in a mortar with 25 ml. distilled water to give a suspension of 3% (w/v) of the drug in a 1% (w/v) gum acacia solution. (On the first day, 0.5% gum acacia solution was used.)

Conduct of Test.—Blood from one of the original isolation rats was diluted with heparinized 5% glucose solution, the dilution being adjusted following a haemocytometer count so that about 1,000,000 motile trypanosomes were present/0.1 ml. Eighty-five mice were each inoculated intraperitoneally with 0.1 ml. of this suspension. Ten uninfected mice were also used. Fresh preparations of the tail blood of infected mice were examined daily (except Sundays); if trypanosomes were not seen in 20 fields of these preparations (magnification about $\times 400$), the mice were classed as negative. Treatment was commenced in all groups on the sixth day after the mice which had been infected received their inocula. At this time all these mice had at least five, and usually more, trypanosomes in each microscopic field of their blood. For treatment, the surviving infected mice were divided at random into four groups of 15 to 17 individuals and one group of five. The uninfected mice were divided into two groups of five. The treatment of these groups is shown in Table I. Nitrofurazone suspension was given intraperitoneally in 10 equal daily doses on the basis of the average body weight of each group of mice: those in group 1 received 0.04 ml. of suspension each; group 2, 0.07 ml.; group 3, 0.14 ml.; and groups 4 and 7, 0.29 ml. The gum acacia solution

TABLE I

DETAILS OF THE ADMINISTRATION AND EFFECT OF NITROFURAZONE SUSPENSION, AND ITS DILUENT ALONE, ON MICE INFECTED WITH *T. RHODESIENSE* AND ON UNINFECTED MICE

The doses indicated in treatment column were given daily for 10 days. * Indicates that only those mice which died with parasitaemia after their treatment had ended (in groups 1 to 4) were included in the calculation. † Indicates inclusion of one mouse which died 4 days after its treatment ended without having relapsed. ‡ Indicates that there were no further deaths in these two groups within the next 42 days.

No. of Group	Treatment	No. of Mice				Survival Time after Infection (Days)*	
		At Start of Treatment	Dying during Treatment	Dying with Parasitaemia after Treatment	Cured (Surviving without Parasitaemia 42 Days after Treatment)	Mean	Range
Mice infected with *T. rhodesiense*							
1	Nitrofurazone, 50 mg./kg.	17	3	14	0	40	34–52
2	,, 100 ,,	16	4	12	0	39	30–49
3	,, 200 ,,	15	7	8	0	52	38–69
4	,, 400 ,,	17	15†	2	0	48	42 and 54
5	1% gum acacia, 0·3 ml. ..	5	2	3	0	19	13–27
Uninfected mice							
6	1% gum acacia, 0·3 ml. ..	5	0‡				
7	Nitrofurazone, 400 mg./kg.	5	2‡				

was given to mice of groups 5 and 6 intraperitoneally in 10 equal daily doses. Group 5 was intended as a control of the virulence of the strain of trypanosomes, group 6 as a control of the toxicity of the gum acacia solution, and group 7 as a control of the toxicity of nitrofurazone to uninfected mice.

RESULTS

The results are shown in Table I. The drug cleared trypanosomes from the blood of all treated mice after two or three doses, but the mice which survived the course of treatment redeveloped parasitaemia between 6 and 23 days later (except one mouse in group 4, which died without having relapsed 4 days after its treatment ended) and subsequently died with high parasitaemia. However, the mean survival times of all groups of treated mice were increased as compared with the untreated infected mice of group 5. Two uninfected mice of group 7 died during their course of treatment presumably due to poisoning by the drug.

DISCUSSION

It is clear from Table I that nitrofurazone, given in ten equal daily doses of 50 to 400 mg./kg., failed to cure any mice infected with a recently isolated strain of *T. rhodesiense*. The infections were, however, temporarily suppressed, and the treated mice survived longer than the untreated controls.

The maximum dosage given in this experiment was apparently lethal to two of five uninfected mice in group 7, after 4 and 6 doses respectively: the survival of all the mice in group 6 suggests that this toxicity could not have been due to the gum acacia solution in which the drug was suspended. The progressively increasing numbers of mice which died during treatment in groups 1 to 4 suggest that the drug may be more toxic to infected than to uninfected mice. Some of the deaths may have been due to trypanosomiasis, but if this were the only explanation then a higher death rate in the groups given the lower dosages would be expected.

These results differ, as far as curative power is concerned, from those of Evens *et al.* (1957a), who cured 3 out of 30 guinea-pigs infected with *T. rhodesiense* with single intramuscular injections of from 75 to 150 mg./kg. of nitrofurazone. The difference may be due to the different host, to differences in the strains of trypanosomes used, or to the route of administration of the drug. The infections treated by Evens *et al.* (1957a) were more chronic than those used in the present work, the average life of the infected guinea-pigs being about 50 days. As recently pointed out by Ashcroft (1958), the albino mouse may not be a very suitable animal for determining the curative powers of drugs in human trypanosomiasis, but it is often the only animal which can be used in large numbers: the results of such experiments can be, at best, only a guide to the behaviour of the drug in human infections.

If used therapeutically, nitrofurazone would be given orally and not parenterally as it was in this test. However, Giarman (1951) showed that estimates of the effective doses in 50% of the animals were similar whether the drug was given orally or subcutaneously, as regards lethal and curative properties. Packchanian (1955) found similar therapeutic results whether the drug was given orally or intraperitoneally to mice infected with *T. rhodesiense*. Thus the route of administration does not seem to affect the absorption of the drug greatly.

Taking into account the results of Evens *et al.* (1957a) and those reported in the present paper, the use of nitrofurazone in the treatment of human infections with *T. rhodesiense* does not seem very promising. However, it appears to be a relatively non-toxic compound and a small trial of its effect on human cases might be justified.

I am grateful to Dr. D. H. H. Robertson for the strain of *T. rhodesiense*, and to Mr. Henry Owino for his assistance.

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THE CENTRAL VASOMOTOR EFFECTS OF 5-HYDROXYTRYPTAMINE

BY

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In the dog, injection of 5-hydroxytryptamine into the cerebral ventricles caused hypotension, inhibition of the pressor response to occlusion of the carotid artery and inhibition of the pressor or depressor response evoked by electrical stimulation of the central end of the cut vagus. Hypotension and inhibition of the vagal vasomotor response also occurred in dogs in which the carotid sinuses had been denervated and the vagi cut. The site of action was central. Local cerebral vascular changes could not have been responsible for the action. The central vasomotor effects of 5-hydroxytryptamine are mediated through the sympathetic outflow. Implications of these findings are discussed in relation to the effects of intravenous 5-hydroxytryptamine and the mechanism of action of reserpine.

Feldberg and Sherwood (1954) studied the effects of drugs introduced into the ventricular system of cats through an implanted metallic cannula. Using a simple polyethylene cannula for injecting drugs into the lateral cerebral ventricles of dogs, we have studied the central vasomotor effects of 5-hydroxytryptamine in this species.

It is generally believed that the quantity of 5-hydroxytryptamine which penetrates the blood-brain barrier after parenteral administration is too small to produce central effects. However, a measurable rise of brain 5-hydroxytryptamine occurs on parenteral administration into mice (Shore, Pletscher, Tomich, Carlsson, Kuntzman, and Brodie, 1957). In this species an intraperitoneal injection of 5-hydroxytryptamine produces, like reserpine, general depression of muscular activity, sedation, and potentiation of the hypnotic action of barbiturates and ethanol (Shore, Silver, and Brodie, 1955). A direct inhibition of central synaptic transmission was observed by Marrazzi and Hart (1955) after the intracarotid injection of small doses of 5-hydroxytryptamine into cats, and Malcolm (1958) showed that intracarotid injection of 5-hydroxytryptamine into cats depressed the cortical response evoked by sensory nerve stimulation, but only with large doses. There are a few observations which show that 5-hydroxytryptamine, by a central action, can influence blood pressure and its responses. Bhargava and Borison (1957) found that, in cats, pressor responses elicited by threshold electrical stimulation of the medulla oblongata were depressed by large doses of 5-hydroxytryptamine given intravenously. The effect was similar to that after reserpine. Costa and Aprison (1957) observed in cross-circulation experiments that perfusion of the isolated head with 5-hydroxytryptamine caused a fall of arterial blood pressure in the recipient. A central depressor action of 5-hydroxytryptamine had previously been suggested by Ginzel and Kottegoda (1954) to explain the depressor effect obtained on its injection into the subclavian artery.

The present experiments show that 5-hydroxytryptamine injected into the cerebral ventricles of dogs caused a fall in arterial blood pressure, probably due to inhibition of sympathetic vasoconstrictor tone and, in addition, it inhibited the blood pressure responses produced by carotid occlusion and by central vagal stimulation.

METHODS

Dogs were anaesthetized with 30 mg./kg. of pentobarbitone sodium given intravenously. Usually both vagi were cut in the neck and the animals were maintained on artificial ventilation. Blood pressure was measured by means of a mercury manometer connected to the left carotid artery.

Reflex vasomotor responses were evoked by occlusion of the right common carotid artery or by electrical stimulation of the central end of a cut vagus nerve. A tubular electrode consisting of a 2 cm. long

polyethylene tube of 5 mm. bore in which platinum wires were embedded was used to stimulate the nerve. The stimuli were obtained from a Stoelting Electronic Stimulator delivering rectangular pulses. The nerve was kept under a pool of medicinal liquid paraffin.

Injections into the lateral cerebral ventricles were made through a polyethylene cannula introduced into a ventricle soon after the animal was anaesthetized. The cannula was 5 cm. long and 2 mm. in bore. The tube was passed through the centre of a pliable metal plate and fixed rigidly to the plate with the inner end projecting 1.5 cm. below it. Two holes were provided on the metal plate to screw it on the skull. A suitable incision of the scalp was made to expose the parietal bone. A burr hole (2 mm. in diameter) was made into the bone at a point 5 cm. rostral and 3 cm. lateral to the occipital protuberance. The dura was punctured through the burr hole and the inner end of the cannula was passed anteromedially into the lateral cerebral ventricle. The metal plate was then screwed snugly on the bone. To ensure further against leakage, a paste made of plaster of Paris was used to seal the plate on to the bone. The small amount of cerebral tissue which entered the cannula was sucked out. The correct placing of the cannula was indicated by the appearance of cerebrospinal fluid on suction. Pulsations of the cerebrospinal fluid could be observed through the transparent plastic cannula. Not more than 1 ml. of drug solution was injected into the ventricle at any one time. At the end of the experiment the position of the cannula was confirmed by cutting a coronal section of the head at the level of the cannula.

Effects of intraventricular injection of drugs were observed on the systemic blood pressure. The effect of a drug was considered valid only when followed by recovery to control values.

Freshly prepared solutions of 5-hydroxytryptamine creatinine sulphate (*p*H 6.5) were used.

Results

Blood Pressure Changes after 5-Hydroxytryptamine.—It is well known from the experiments of Page and McCubbin (1953) that, in dogs, an intravenous injection of 5-hydroxytryptamine causes an initial fall followed by a transient rise with finally a secondary prolonged fall in blood pressure. These three phases occurred in the present experiments on intravenous injection of 5-hydroxytryptamine (1 mg. in dogs weighing about 12 kg.). Intraventricular injection of 5-hydroxytryptamine, on the other hand, caused only a prolonged fall in arterial blood pressure. In a dog of about 12 kg., 1 to 2 mg. of 5-hydroxytryptamine caused a fall of 25 to 50 mm. Hg, and 0.5 mg. a fall of about 10 mm. Hg. These average values were calculated from the results of thirty-six injections. A more pronounced depressor effect was obtained with intraventricular 5-hydroxytryptamine when the carotid sinuses were denervated and the vagi cut. Then a dose of 0.5 mg. produced about the same effect as 1 mg. in a dog with normally innervated carotid sinuses.

The depressor action of intraventricular 5-hydroxytryptamine is probably due to central inhibition of sympathetic vasoconstrictor tone, since it no longer occurred when the autonomic

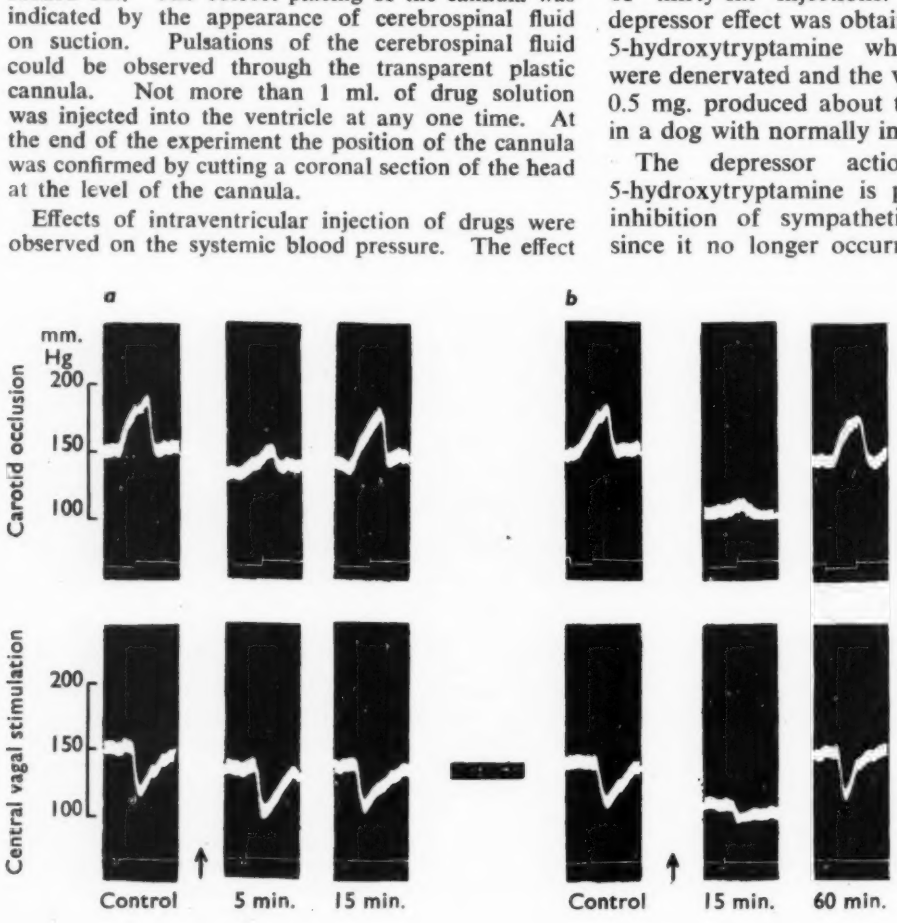

FIG. 1.—Dog, 12.5 kg. Blood pressure records. Comparison of effects of, a, intravenous and, b, intraventricular injections of 1 mg. of 5-hydroxytryptamine respectively at the arrows on the pressor response to carotid occlusion (upper records) and depressor response to electrical stimulation (30 V., 240 sec.) of afferent vagus nerve (lower records). Note the marked fall in blood pressure and the inhibition of both vasomotor responses on intraventricular administration at 15 min. Time, 1 min.

ganglia were blocked by an intravenous injection of 1 mg./kg. of pentolinium or when the vessels were rendered insensitive to the transmitter of the adrenergic vasoconstrictor fibres by the intravenous injection of 2 mg./kg. of hydrallazine. The presence of ganglionic block after pentolinium was confirmed by the absence of retraction of the nictitating membrane in response to electrical stimulation of the preganglionic cervical sympathetic nerve.

The centrally-induced hypotension produced by intraventricular 5-hydroxytryptamine is unlikely to be due to local vasoconstriction of cerebral vessels, since certain vasoconstrictor and vasodilator agents, when given intraventricularly. did not significantly change the arterial blood pressure nor did they modify the vascular reflexes. The substances tested were noradrenaline (5 μg. to 1 mg.), vasopressin (2 to 4 units) and histamine (50 to 750 μg.).

Effects of 5-Hydroxytryptamine on Vascular Reflexes.—Fig. 1 shows the effect of 1 mg. of 5-hydroxytryptamine given intravenously and intraventricularly in the same dog on the pressor response to occlusion of the carotid artery and on the depressor response to central vagal stimulation. During the secondary depressor phase, about 5 min. after the intravenous injection, the pressor response to the occlusion of the carotid was greatly reduced, but the depressor response to central vagal stimulation was unchanged. About 15 min. after the injection, the pressor response to occlusion of the carotid had recovered. When both tests were carried out about 15 min. after the intraventricular injection when the blood pressure was still low, both the pressor response to carotid occlusion and the depressor response to central vagal stimulation were abolished. Recovery of both responses was complete in 1 hr.

When the carotid sinuses were denervated and the vagi cut, electrical stimulation of the central end of the left vagus elicited a pressor response which was greatly reduced when the vagus was again stimulated 15 min. after an intraventricular injection of 0.5 mg. of 5-hydroxytryptamine. Recovery was complete after 1 hr. (Fig. 2).

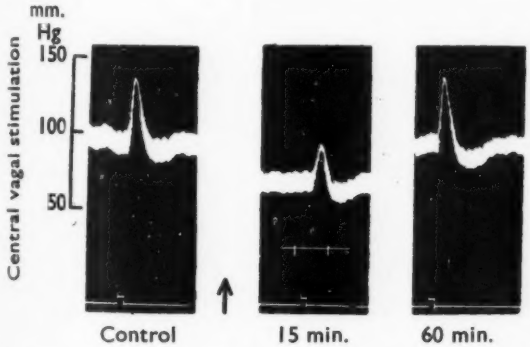

FIG. 2.—Dog, 12.0 kg. Carotid sinus and vagus nerves cut. Record of blood pressure. Effect of intraventricular injection of 0.5 mg. of 5-hydroxytryptamine at the arrow on pressor response to electrical stimulation (7 V., 60/sec.) of central cut end of vagus nerve. Note the fall in blood pressure and diminution of the vagal response at 15 min. Recovery complete at 60 min. Time, 1 min.

DISCUSSION

Ginzel (1958) failed to observe vasomotor effects on intraventricular injection of 5-hydroxytryptamine into cats in doses comparable to those employed in the present experiments on dogs. This may be due to a species difference. In dogs, hypotension occurs regularly on intraventricular injection of 5-hydroxytryptamine but only with relatively high doses. These doses abolish reflex pressor and depressor responses. A direct neuronal action of 5-hydroxytryptamine is assumed to be responsible for these central effects. The existence of a local vasoconstrictor action on the brain vessels is unlikely because intraventricular noradrenaline, vasopressin and histamine did not influence the arterial blood pressure nor did they affect the vascular reflexes.

The effects of intraventricular 5-hydroxytryptamine, namely the hypotension as well as the abolition of the reflex blood pressure responses to carotid occlusion and to central vagal stimulation, suggest depression at the brain stem level, probably on the vasomotor centres. That these effects no longer occur when the autonomic ganglia are blocked or the vessel wall is rendered insensitive to the mediator of adrenergic sympathetic fibres by hydrallazine shows that the depressant effect of 5-hydroxytryptamine is exerted via the sympathetic outflow.

After intravenous injection of 5-hydroxytryptamine the response to central vagal stimulation remained unimpaired, whereas the response to carotid occlusion was greatly reduced. It is unlikely that this effect of 5-hydroxytryptamine is also due to central depression, because it would then be difficult to explain the unchanged response to central vagal stimulation. The depression of the response to carotid occlusion on intravenous injection of 5-hydroxytryptamine is probably due to its action on the carotid sinus receptors which are known to be vulnerable to this drug when given intravenously (Ginzel and Kottegoda, 1954).

Centrally-induced inhibition of sympathetic vasoconstrictor tone, which appears to be the

cause of the prolonged fall in arterial pressure on intraventricular injection of 5-hydroxytryptamine, may well produce the secondary prolonged hypotension when this substance is injected intravenously. This hypotension has never been satisfactorily explained and, although it is generally assumed to be due to a peripheral action of 5-hydroxytryptamine, the evidence for this assumption is anything but convincing. Page and McCubbin (1956) attributed this hypotension to peripheral inhibition of neurogenic vasoconstrictor tone. They were able to block the effect with large doses of promethazine and thus considered release of endogenous histamine to be a contributory factor in the causation of the hypotension. However, promethazine in large doses also abolishes adrenergic nerve effects (Gengoux, 1956). On the assumption that the hypotension results from centrally-induced inhibition of vasoconstrictor tone this property of promethazine would also explain why it prevents the hypotension. Furthermore, histamine release has actually been excluded as a possible mechanism since Reid (1952) found that the hypotension was not prevented by diphenhydramine or mepyramine. In his opinion, the cause for the hypotension has not been satisfactorily elucidated, but by elimination he assumes that it may result from impaired performance of the cardiac muscle as a result of coronary arterial constriction. It seems to us more likely that central inhibition of vasoconstrictor tone is responsible for both the hypotension after intraventricular injection of 5-hydroxytryptamine and the secondary prolonged hypotension after its intravenous injection. This view is strengthened by the fact that a centrally-induced hypotension is also observed when the 5-hydroxytryptamine is injected into the subclavian artery (Ginzel and Kottegoda, 1954) or when the head is perfused with it in cross-circulation experiments (Costa and Aprison, 1957).

Reserpine is known to release 5-hydroxytryptamine from the brain in addition to other sites (Pletscher, Shore and Brodie, 1956) and the hypotension produced by reserpine has been shown to correspond well with this action (Deming, Bogdanski, Udenfriend, Shore and Brodie, 1956). That 5-hydroxytryptamine in the cerebrospinal fluid is capable of inducing hypotension by a central action suggests the possibility that, when reserpine releases 5-hydroxytryptamine from the brain, the free 5-hydroxytryptamine acts directly on the vasomotor centre and depresses it.

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DRUG ANTAGONISM AND AUDIOGENIC SEIZURES IN MICE

BY

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The occurrence of audiogenic seizures in mice has been used as the basis of a method for measuring the antagonism between sodium thiopentone and bemegride or leptazol. Thiopentone in doses of 8 and 16 mg./kg. protected all the mice against the occurrence of audiogenic seizures. Bemegride and leptazol were administered in doses having molecular concentrations which bore a simple relationship to the doses of thiopentone. Bemegride was four times as powerful as leptazol in antagonizing the protective effect of thiopentone, and comparison of their regression lines showed that both analeptics acted in the same way. The antagonistic effect of bemegride occurred with doses less than its normal convulsant dose, but the doses of leptazol needed to antagonize thiopentone were within the convulsant range, and it increased the severity of any audiogenic seizures.

The susceptibility of rats and mice to audiogenic seizures has been known for a considerable time, and has been fully described (Smith, 1941 ; Frings and Frings, 1953). The psychological and physiological factors which influence the occurrence of these seizures have also been extensively investigated. The audiogenic seizures have been compared with human epilepsy (Lindsley, Finger, and Henry, 1942) and with leptazol convulsions (Maier and Sacks, 1941). Like seizures induced by electric shock, audiogenic seizures are produced by physical stimuli, and lend themselves easily to inhibition by hypnotic and anticonvulsant drugs. There are quantitative differences in the power of these drugs to suppress the seizures, and Lehmann, Halpern, and Busnel, (1957) and Busnel, Lehmann, and Busnel (1958) have suggested sites at which the drugs might act.

Previous investigators have studied the actions of convulsant or anticonvulsant drugs individually on the production of audiogenic seizures. In the present experiments the ease and certainty with which audiogenic seizures can be produced in susceptible mice have been used as the basis of a method for measuring the antagonism between anticonvulsant and convulsant drugs. The occurrence of an audiogenic seizure has been used to determine whether the action of an anticonvulsant drug, which inhibits the production of a seizure, has been overcome by the action of an antagonist Thiopentone has been used as the anticonvulsant, and leptazol and bemegride have been used as its antagonists. Leptazol and bemegride themselves have convulsant actions, but, by comparing their convulsant doses with the doses necessary to antagonize the action of thiopentone, it was possible to differentiate these two effects. In order to make these dose comparisons, the drugs have been administered in doses which bore simple relationships to their molecular weights.

METHODS

Male and female mice (18 to 35 g. body weight) were derived from the strain of Swiss albino mice selected by Frings and Frings (1953) in the U.S.A. These mice respond to a certain kind of intense auditory stimulation by seizures of a clonic-tonic type, named audiogenic seizures (Frings and Frings, 1952).

Audiogenic seizures were produced by an auditory stimulus of 10 kc./sec. with an intensity of 100 decibels emitted close to the mice. The mice were tested individually (Lehmann, 1956). The audiogenic seizure started after a short latent period of 5 to 40 sec. and was indicated by flushing of the tail and exophthalmos, immediately followed by considerable agitation during which the mouse ran rapidly round the cage. Generalized clonic convulsions then occurred, which gradually disappeared or were followed by a catatonic seizure, during which the mouse became stiff and breathing ceased. Breathing

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generally recommenced spontaneously in about 30 sec. and the mouse recovered. Occasionally artificial ventilation was necessary, but it was uncommon for death to follow the seizure. This sequence of events lasted about 1 min., and the mouse remained resistant to further auditory stimuli for a period of 6 to 12 hr. In assessing the results, an audiogenic seizure was defined as a seizure in which clonic or clonic-tonic phases occurred. The phases of vasodilatation, exophthalmos, and running, when they occurred alone, were not classified as audiogenic seizures. The ability of the mice to develop seizures was tested 24 hr. before the administration of the drugs, during the period of action of the drugs, and 24 to 48 hr. after administration. Any animals in which audiogenic seizures did not occur during the experiments done before and after the 24 hr. test period were excluded from the results. The experiments were always performed in the same laboratory, the temperature of which varied between 19.8 and 21.0°.

Sodium thiopentone, leptazol, and bemegride were administered by intraperitoneal injection. Dilutions were made in 0.9% NaCl, and the volume injected never exceeded 0.75 ml. Thiopentone was administered at zero time, and the reactions of the mice to the auditory stimuli were tested 30 min. later. When an analeptic was being examined together with the thiopentone, the analeptic was injected 10 min. after the thiopentone, and susceptibility to the stimulus was tested 20 min. later. At each end of the analeptic dose ranges four mice were used, and for the other doses larger groups (up to twelve mice) were tested.

Results

The Protective Action of Thiopentone

Thiopentone in doses of 8 or 16 mg./kg. abolished audiogenic seizures 30 min. after administration in all the mice. Neither of these doses caused loss of consciousness or any obvious signs that they were having a therapeutic effect. Smaller doses had less protective action, and a dose of 1 mg./kg. did not prevent the occurrence of seizures. The protective effect of thiopentone is shown in Fig. 1.

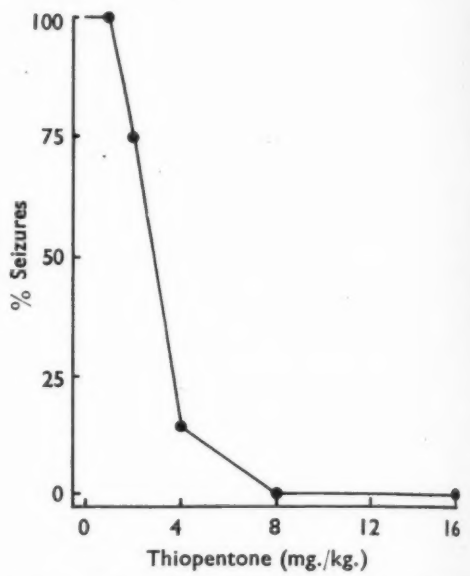

FIG. 1.—The effect of thiopentone in preventing the occurrence of audiogenic seizures in mice.

TABLE I

PERCENTAGE OF MICE HAVING CONVULSIONS WITHIN 10 MIN. OF THE INTRAPERITONEAL INJECTION OF BEMEGRIDE AND OF LEPTAZOL

Drug	Dose (mg./kg.)	No. of Mice	% of Mice having Convulsions	
			Clonic	Tonic
Bemegride	20	2	0	0
	25	6	40	0
	30	5	100	0
	40	4	100	100
Leptazol	40	5	0	0
	50	6	50	17
	60	5	100	50

The Convulsant Effects of Bemegride and Leptazol

The convulsions produced by these drugs were similar. They occurred within 2 to 10 min. of administration, and, with the smaller doses, consisted of intermittent clonic convulsions. With larger doses, the clonic phase ultimately developed into a tonic phase, which was invariably fatal. The doses which caused the convulsions are shown in Table I. The dose of bemegride to cause convulsions in 50% of the mice (CD50) was obtained by interpolation of the line relating log dose and percentage of mice convulsing; it was found to be approximately 25.5 mg./kg. The CD50 of leptazol was 50 mg./kg.

Effects of the Analeptics and Thiopentone on Audiogenic Seizures

The method, described by Sanford (1958) for cats, in which the relationship between the doses of the hypnotic and analeptic drugs is expressed in terms of molecular ratios, has been employed for the quantitative assessment of drug antagonism in the present experiments. Doses of thiopentone were used which prevented the occurrence of audiogenic seizures in all the mice—namely, 8 and 16 mg./kg. The analeptics were administered in doses which bore simple molecular relationships to these doses of thiopentone. When referring to the doses of the analeptics the term molecular dose

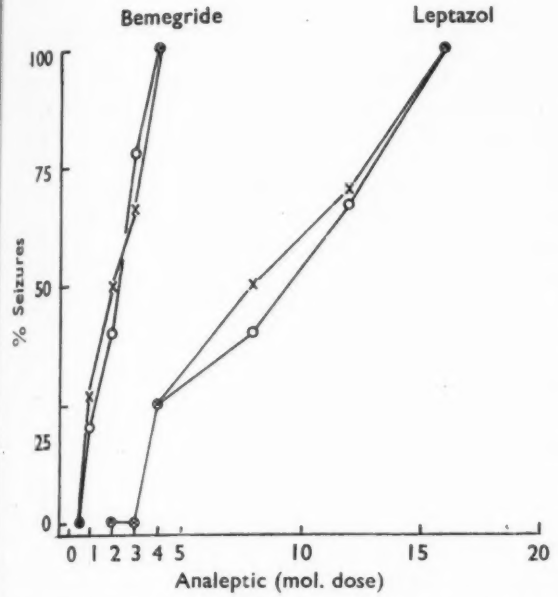

FIG. 2.—The actions of bemegride and leptazol in restoring the susceptibility to audiogenic seizures in mice pretreated with thiopentone. X—X, thiopentone 8 mg./kg. O—O, thiopentone 16 mg./kg.

(mol. dose) has been employed. It indicates the number of molecules of the analeptics administered per single molecule of the hypnotic.

Bemegride.—The administration of bemegride to mice which had received thiopentone in doses of either 8 or 16 mg./kg. caused the reappearance of the susceptibility to audiogenic seizures. Equimolar doses of bemegride were equally effective against the large and the small dose of thiopentone. A 0.5 mol. dose did not cause the reappearance of this susceptibility with either dose of thiopentone, 2 mol. doses caused the reappearance of susceptibility to seizures in 50% of the mice, and 4 mol. doses completely abolished the protective effect of the thiopentone (Fig. 2).

Transformation of the experimental results into probits for each dose of thiopentone, and analysis of the regression lines thus obtained, showed that a linear response/log dose relation existed. A test for parallelism between the two lines demonstrated that they were parallel, and that there was no significant difference between their positions. Therefore the experimental results for the antagonistic action of bemegride towards both doses of thiopentone have been combined and a common regression line has been fitted (Fig. 3).

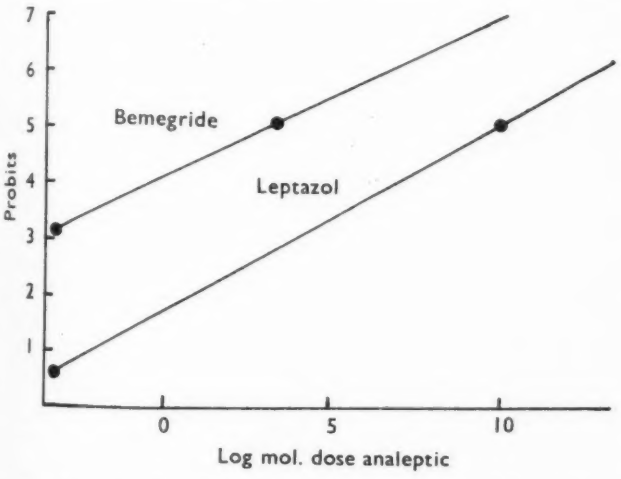

FIG. 3.—Regression lines showing the ability of leptazol and bemegride to restore the susceptibility to audiogenic seizures of mice pretreated with thiopentone. Each line is obtained from the values of the analeptic in the presence of 8 and 16 mg./kg. of thiopentone. Abscissa, log mol. dose. Ordinate, probits of mice showing audiogenic seizures.

TABLE II
COMPARISON OF THE CONVULSANT DOSES (CD50) AND THE AUDIOGENIC SEIZURE DOSES (ASD50) OF BEMEGRIDE AND LEPTAZOL

The ASD50 is the dose of analeptic with which, in the presence of thiopentone, 50% of the animals had audiogenic seizures.

Analeptic	CD50 (mg./kg.)	ASD50	
		8 mg./kg. Thiopentone	16 mg./kg. Thiopentone
Bemegride ..	25·5	9·1	18·2
Leptazol ..	50·0	31·9	63·7

Mice which had received the 4 mol. doses of bemegride in the presence of thiopentone did not appear to be abnormally sensitive to sensory stimuli, and did not show the periodic clonic seizures which occurred when they received bemegride alone in similar doses. In Table II the CD50 of bemegride by itself is compared with the dose which would cause audiogenic seizures in 50% of the mice (ASD50) in the presence of thiopentone. The values of the ASD50 have been expressed as metric doses in Table II. It can be seen that the reappearance of audiogenic seizures in the mice occurred after doses of bemegride smaller than those necessary to produce convulsions when the drug was acting alone.

Leptazol.—Leptazol (2 to 16 mol. doses) also caused the reappearance of susceptibility to audiogenic seizures in mice which had received both 8 and 16 mg./kg. of thiopentone. With 16 mol. doses of leptazol seizures reappeared in all the mice. A 2 mol. dose had no effect, and an 8 mol. dose caused the reappearance of seizures in about half of the mice (Fig. 2). Transformation

of the results into probits showed that linear response/log dose relations existed for each dose of thiopentone, and that the lines corresponded closely. As with bemegride, a common regression line was fitted to the results (Fig. 3).

Mice which had received the 16 mol. dose became restless, and sensitive to external stimuli, but did not develop convulsions spontaneously. When subjected to the auditory stimulus they quickly developed severe seizures of the clonic-tonic type, and these were fatal in 10% of the animals. It was clear that the large dose of leptazol increased the severity of the audiogenic seizures. It can be seen from Table II that a larger dose of leptazol than the CD50 was necessary to antagonize the 16 mg./kg. dose of thiopentone.

Comparison of the Effects of Bemegride and Leptazol

The regression lines for bemegride and leptazol in Fig. 3 were tested and shown to be parallel. The ASD50 of bemegride against both doses of thiopentone was 1.929 mol. doses, and that for leptazol was 7.658 mol. doses, giving an ASD potency ratio of 3.970. By calculation the ASD potency ratio of the two drugs was found to be 4.000 (fiducial limits 2.914 and 5.943, $P=0.05$). Leptazol, besides being less potent than bemegride in antagonizing thiopentone, was an effective antagonist only in doses close to or exceeding its convulsant dose.

Discussion

Several methods have been used to investigate drugs which appear to have antagonistic actions in the central nervous system. The ability of analeptics to prevent the lethal effects of a hypnotic (Maloney, 1931), to restore breathing (Marshall, Walzl, and Le Messurier, 1937), to shorten the sleeping time (Shaw, Simon, Cass, Shulman, Anstee, and Nelson, 1954), and to influence muscular activity in conscious mice (Dews, 1953 ; Brown, 1957), can all be used to measure the effects of drugs on functions attributed to extensive or more circumscribed areas of the central nervous system. The present experiments have examined the antagonistic actions of drugs on the areas of the central nervous system in which convulsions originate. However, it is clear that these areas, which functionally might be fairly circumscribed, display considerable variation in their sensitivity to different convulsant and anticonvulsant drugs (Tripod, Bein, and Meier, 1954). In the present experiments, thiopentone prevented the occurrence of the seizures in doses which had no other noticeable effects on the mice. Mercier (1950) and Gross, Tripod, and Meier (1955) found that phenobarbitone was effective against audiogenic seizures in small doses which caused few other effects, and Humphrey (1942) comments on the very small dose of atropine, in comparison with the lethal dose, which was needed to prevent the occurrence of audiogenic seizures. It is clear from these results that the areas in which convulsions arise are more sensitive than other parts of the brain to barbiturates and other hypnotic drugs. Halpern and Lehmann (1956) explained these results, and their own in which the carbamate of methylpentynol inhibited audiogenic seizures, by suggesting that the seizures are mediated through the reticular formation.

The analeptics were always given at the same interval after the barbiturate, and in making quantitative comparisons it has been assumed that the analeptics reached their sites of action in the brain in the same ratios as those in which they were injected. Both bemegride and leptazol antagonized the protective action of thiopentone against audiogenic seizures. The occurrence of a seizure in the present experiments has been used as an indication of complete antagonism of the barbiturate by each of the analeptics. Leptazol increased the severity of the audiogenic seizures in doses which corresponded approximately to the convulsant dose, and also to the dose which antagonized the effect of the thiopentone on the site of origin of convulsions. The ability of leptazol to increase the severity of the audiogenic seizures has also been observed by other workers (Maier, Sacks, and Glaser, 1941 ; Karn, Lodowski, and Patton, 1941 ; Chen, Ensor, and Bohner, 1954). The 4 mol. doses of bemegride, both equally effective in antagonizing thiopentone, were less than the convulsant dose of bemegride. Quantitative comparison of the antagonism of the analeptics towards thiopentone showed that bemegride was four times as powerful as leptazol. Bemegride therefore acted as an effective antagonist to thiopentone in a dose which, molecule for molecule, was smaller than the antagonistic dose of leptazol. This dose was also considerably less than the normal convulsant dose.

I should like to express my thanks to Dr. B. N. Halpern for his interest and encouragement throughout these experiments, to Monsieur R. G. Busnel and his staff for their interest, hospitality, and assistance, and to Mr. R. L. Plackett for his guidance and help in the statistical analysis of the results. This work was performed during the tenure in France of a Wellcome Research Travel Grant.

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THE INEFFECTIVENESS OF (±)-CARNITINE PREVENTING THE TWITCHINGS OF STRIATED FROG MUSCLE IN 0.7% SODIUM CHLORIDE SOLUTION

BY

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The spontaneous twitchings of isolated frog sartorius muscles in 0.7% NaCl solution have been studied. Addition of 1 mg./ml. of (±)-carnitine hydrochloride, or of (±)-carnitine base, to the bath fluid had no influence on the spontaneous activity of the muscles, their excitability or their ability to liberate potassium. This indicates that carnitine is not a natural inhibitor of striated frog muscle. Fluids enriched with potassium either from twitching muscle or by addition of KCl inhibited the activity of muscles reversibly.

The wide distribution of carnitine in biological materials, especially in skeletal muscle, has been known for over 50 years (Gulewitsch and Krimberg, 1905). Despite many investigations of the physiological properties of this substance (Weger, 1936; Strack and Försterling, 1953; Fraenkel, 1954) its function in muscle is unknown. Hayashi (1956), however, allocated to carnitine an important rôle in his hypothesis concerning the chemical physiology of excitation in muscle and excitable tissues.

Hayashi (1956) studied the rhythmic contractions of frog sartorius muscle immersed in a solution of isotonic NaCl (Mines, 1908; Adrian and Gelfan, 1933). He observed that, when a freshly excised muscle is put into the bath fluid in which another muscle had previously been immersed, it failed to exhibit rhythmic contractions. He concluded that the bath fluid contained a factor which inhibited movements, and having detected carnitine in the solution suggested that this was the inhibiting factor; he called it "inhibitine."

The theory of Hayashi (1956) was mentioned in several recent reviews (for example, Purpura, Girado, Smith, Callan, and Grundfest, 1959), and it was considered of interest to confirm two of his basic experiments, namely the appearance of an inhibiting factor in the bath fluid of a twitching muscle and the inhibiting effect of carnitine, prepared synthetically, on the twitching muscle. In addition the influence of carnitine on muscular excitability and on the liberation of potassium during immersion of the muscle in saline was studied.

METHODS

Two samples of carnitine hydrochloride were used; one was received from Dr. Fraenkel and the other from Dr. Deltour. They contained crystals of different sizes but of the same melting point. Carnitine base (synthesized by Dr. Deltour; m.p. 201 to 203°, free of chloride) had not more than 2% of impurities. A modified Ringer solution of the following composition was used: NaCl, 111 mM.; KCl, 2.7 mM.; $CaCl_2$, 1.1 mM.; NaH_2PO_4, 0.08 mM.; $NaHCO_3$, 2.4 mM.; glucose, 11 mM. The *p*H of this solution was 7.2.

Rana temporaria (male and female) were obtained from Rheinland water meadows. Experiments were done from January to April in two successive years. In all experiments the temperature was kept at 20° (±1°). When twitching was studied by visual observation, both sartorii were dissected from tendon to tendon. For mechanical recording of the twitches the pubic symphysis was cut under a binocular microscope and each muscle was left attached to a splinter of bone. After dissection, the muscles were immediately suspended in calcium-free Ringer solution for 45 sec. by a thread tied to the tendon. Finally, the muscles were placed in test-tubes containing 5 ml. of bath fluid. One muscle of each pair was put into the test fluid, the other was used as a control.

The action of various solutions on the twitching induced by exposure to NaCl was studied visually to see if twitching was suppressed in the treated muscle. The observation periods varied from 30 min. to 3 hr. A rough quantitative idea of the effectiveness of the

suppression was obtained by attaching the muscle to the moving arm of a condenser microphone (Brecht and Boucke, 1953) by means of a thread tied to the tendon, the pelvic end being fixed. The output from the microphone was fed into a conventional electroencephalogram amplifier feeding into a pen recorder. Excitability was tested directly by stimulating the muscles with rectangular current pulses of 2 msec. duration at 1 sec. intervals, and with current strengths of up to 100 mA. The electrodes were two silver wires of 0.5 mm. diameter, set 2 mm. apart in plastic ground down to expose the wires for a distance corresponding to the width of a muscle. They were placed under the muscle at its pelvic end. Excitability measurements were only used to give a general idea of the onset of conduction block.

Potassium was analysed with a Beckman flame photometer, Model D.U.

Results

The left-hand muscles of a pair were placed in the fluid in which other muscles (100 to 500 mg. wet weight) had previously been immersed for several hours until they stopped twitching. The corresponding right-hand muscles were put into fresh 0.7% saline. Left-hand muscles, immersed in a bath which had contained previously a muscle of approximately 100 mg., had activities similar to that of the corresponding control muscle. In nine experiments, muscles were immersed in fluid in which several muscles (about 500 mg.) had previously twitched (referred to as "used" fluid). In these experiments twitching was inhibited, though not completely suppressed. The potassium contents of "used" fluids which had contained 530, 580, and 630 mg. of fresh muscle for 6 hr. were found to be 2.4, 2.3, and 2.6 mM. KCl respectively. If these muscles were removed from this potassium enriched saline they commenced twitching once again.

Starting from the above observation that enrichment of 0.7% saline by KCl is accompanied by depression of muscular activity, 64 left-hand muscles were immersed in 0.7% saline and their corresponding right-hand muscles in 0.7% saline containing additional KCl (0.67–2.7 mM.). Some depression of activity was observed in 21 of 25 experiments with a concentration of 1.3 mM.-KCl. With 2 and 2.7 mM.-KCl, twitching was strongly depressed in 30 of 34 experiments immediately after transferring the muscles into the solution containing KCl, but some activity remained during the observation period of 3 hr. The behaviour of these muscles was similar to that of muscles placed in "used" saline.

The action of carnitine itself was studied on 27 muscle pairs using two samples of (±)-carnitine hydrochloride or carnitine base at bath concentrations of 125, 250, 500, or 1,000 μg./ml. in 0.7% saline. Carnitine hydrochloride solutions are strongly acid. Therefore the *p*H was usually brought to 7.5, but in some experiments it was varied from 4 to 9 by the addition of 0.1 M NaOH. Changes of *p*H in this range were found to have no influence on the twitching. Pairs of muscles were first suspended for 45 to 60 min. in 0.7% saline to assess their normal response. One of each pair was then transferred to the bath containing carnitine and the other to fresh 0.7% saline and both were observed for a further 30 min. or more. Neither carnitine hydrochloride nor carnitine base inhibited twitching.

The excitability of muscle suspended in Ringer solution was tested at various stimulus strengths, but no change occurred when 1 mg./ml. carnitine hydrochloride or carnitine base was added. Likewise there was no appreciable effect of carnitine on the rate of liberation of potassium from muscles in 0.7% saline (Table I).

TABLE I

ACTION OF CARNITINE ON THE RATE OF LIBERATION OF POTASSIUM

Frog sartorii muscles (right-hand) in 0.7% saline for 3 hr.; the corresponding left-hand muscles in the same solution with carnitine (1 mg./ml.).

	Potassium in Bath (μg./100 mg. of Muscle)			
With carnitine ..	53	56	62	71
Without ,, ..	55	58	73	67

Discussion

The highest concentrations of carnitine found by Fraenkel (1954) and other workers in mammalian tissue were 150 to 450 μg./g. of fresh muscle. The carnitine content of frog muscle, therefore, would not be likely to exceed 100 to 500 μg./g. Hayashi (1956) found that 500 mg. of frog muscle immersed in 5 ml. of 0.7% saline for 6 to 7 hr. produced enough "inhibitine" to suppress the twitching. Such inhibition corresponded in his experiments to the effect of 24 to 48 μg. of extracted "inhibitine"/ml. or 240–480 μg. of carnitine/ml., as synthesized by himself. The carnitine content which he found necessary to produce an inhibition, therefore, exceeds the quantity which a frog muscle might be expected to release. In the present experiments a concentration of 1,000 μg./ml. of synthetic carnitine had no effect on the twitch activity of the muscles, on their excitability or on their ability to liberate potassium.

Hayashi (1956) prepared his carnitine by a method ascribed to Tomita (Hayashi, 1956).

Bhattaharyya (quoted by Friedman, Galun, and Fraenkel, 1957) tried to use this method, but his attempts to duplicate the results of Tomita, or achieve the same results by other chemical means, consistently failed. It seems possible, therefore, that the attempts of Hayashi (1956) to produce carnitine led either to a substance different from carnitine or that his carnitine contained an impurity which itself had a high inhibiting effect.

The inhibiting factor appearing in the fluid of a twitching muscle may be the potassium which has diffused out of the muscle fibres (Dulière and Horton, 1929). Ringer (1886) added KCl (100 μg./ml. in 0.7% saline) to the bath and observed that the contractions all but ceased. Our results show that additions of 1.4–2.7 mM.-KCl to the saline reduced the activity reversibly. Therefore, the potassium content of a "used" fluid, found by Hayashi (1956) to be equivalent to 1.4 mM.-KCl, could be sufficient to produce a depression.

I am indebted to Professor J. H. Gaddum for the hospitality of his department and for his advice and interest. I am also indebted to Dr. Fraenkel and Dr. Deltour for kindly giving me samples of carnitine. The work was done during the tenure of a Rike Fellowship from the International Pharmacological Society, 1957/58.

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DRUG RESISTANCE IN TRYPANOSOMES; CROSS-RESISTANCE ANALYSES

BY

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(RECEIVED APRIL 14, 1959)

Eight strains of *Trypanosoma rhodesiense*, made resistant respectively to atoxyl, butarsen, acriflavine, stilbamidine, Surfen C, suramin, and pontamine sky blue 5BX, have been examined for cross-resistance to representatives of nine structurally dissimilar groups of trypanocide. On the basis of their predominant ionic form at blood *p*H, these groups are considered in three main classes: (*a*) feebly ionized (neutral aromatic arsenicals), (*b*) ionized as cations (melaminyl arsenicals and antimonials, acridine derivatives, diguanidines and diamidines, 6-aminoquinoline and 6-aminocinnoline derivatives, phenanthridinium derivatives, triphenylmethane dyes), and (*c*) ionized as anions (carboxylated aromatic arsenicals and sulphonated naphthylamine derivatives). The results are discussed in relation to those of other workers and to possible modes of trypanocidal drug action. Cross-resistance behaviour is not wholly explicable on an ionic basis; the results suggest that stereospecific structural changes associated with initial drug uptake occur in resistant trypanosomes.

The novel structure and properties of the "melamine" class of arsenical and antimonial trypanocides introduced by Friedheim (Friedheim, 1939, 1940, 1944; Friedheim and Berman, 1946; Friedheim, Vogel, and Berman, 1947) led us to produce a strain of *Trypanosoma rhodesiense* resistant to melarsen (disodium *p*-melaminylphenylarsonate), a representative member of the new drug group. We found that this strain was unexpectedly, and almost totally, resistant also to trypanocidal diguanidines and diamidines (Rollo and Williamson, 1951). Subsequently we tested a wider variety of trypanocides against this and five other different resistant strains (Williamson and Rollo, 1952; Goodwin and Rollo, 1955), using these "therapeutic sieves," in Ehrlich's analogy, to sift out any common factors of activity not otherwise apparent among unrelated drug types. During this latter survey, we showed that a stilbamidine-resistant strain (Fulton and Grant, 1955) was cross-resistant to the "melamine" trypanocides, thus demonstrating a reciprocal resistance relationship between the "melamine" and diamidine classes of drug.

One possible mechanism for trypanocidal drug resistance can be based on the familiar assumption that there are at least two stages in drug action, a preliminary fixation and a subsequent lethal action, of which the former is modified in the resistant trypanosome so that the drug is no longer taken up (Clark, 1933; King and Strangeways, 1942). This is essentially the fifty-year-old *chemorezeptor* hypothesis of Ehrlich which, as Schnitzer and Grunberg (1957) indicate, "has not been replaced by either a more lucid or more comprehensive working hypothesis." We have attempted to elaborate this hypothesis with evidence from experiments on selective interference with trypanocidal action, the effect of *p*H and oxidation-reduction potential on trypanocidal action *in vitro*, the activity of selective enzyme inhibitors on normal and resistant trypanosomes *in vitro* and the physical and chemical properties of homogenates of normal and resistant trypanosomes. The present paper concerns the production and cross-resistance behaviour of a number of drug-fast strains, several of which have not hitherto been described.

METHODS

Trypanosome Strains

All the resistant strains described here are offshoots of a parent strain of *T. rhodesiense* (Yorke, Adams, and Murgatroyd, 1929) isolated from man in 1923 and subsequently maintained by blood passage in mice. Dr. J. D. Fulton, of the National Institute

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for Medical Research, very kindly provided us with the stilbamidine-resistant strain; the other resistant strains were obtained as follows:

Atoxyl (sodium p-aminophenylarsonate)-antimony potassium tartrate(tartar emetic)-fast.—This was developed 28 years ago by Yorke and Murgatroyd (1930); its resistance is still unchanged.

Suramin-fast.—This was developed in 1937 to 1938 by Dr. F. Hawking in the Liverpool School of Tropical Medicine and subsequently treated by Fulton and Yorke (1941). Its resistance was maintained unimpaired up to the most recent time of examination (1953 to 1954).

Melarsen-, butarsen-, and Surfen C-fast strains.—These were developed by us in the Liverpool School of Tropical Medicine (1948 to 1949) and re-established subsequently by one of us (J. W.) in the London School of Hygiene and Tropical Medicine and in the West African Institute for Trypanosomiasis Research Laboratories at Vom, Nigeria, where the acriflavine- and pontamine sky blue 5BX-fast strains were also developed.

All resistant strains were produced by intraperitoneal injection of a minimal effective dose of drug into infected mice or rats, followed by blood passage of the relapse strain and successive repetition of this procedure, increasing the dose where necessary as the strain developed resistance.

Drug Response Tests

These were made with batches of five or more mice of approximately 20 g. body weight, each given a single intraperitoneal drug injection (0.5 ml./20 g.) 48 hr. after intraperitoneal infection with approximately 20,000 trypanosomes; at this stage microscopic examination of blood showed one to ten parasites per field (objective 1/6 in., ocular ×10). Under these conditions, the terms "minimal effective dose" (ED90), "minimal curative dose" (CD90), and "maximum tolerated dose" (LD10), as used here and in the accompanying tables, are defined as:

ED90: That dose of drug which clears parasites from the peripheral blood for at least 24 hr. in at least 90% of infected mice, no trypanosomes being visible in thirty random microscope fields of a coverslip preparation of a drop of tail blood.

CD90: That dose which clears parasites from the peripheral blood for at least 28 days in at least 90% of infected mice.

LD10: That dose which kills not more than 10% of treated mice. Confidence limits of these doses for a 0.95 probability lie approximately in the range 70 to 150%, calculated by the method of Litchfield and Wilcoxon (1949).

Trypanocidal Action in Vitro

This was measured by the technique of Yorke and Murgatroyd (1930). Trypanosomes from infected rat blood were washed free of red cells and plasma and resuspended in glass-capped Kahn tubes at a concentration of 10^6/ml. in a sterile medium consisting of equal parts of rabbit serum and glucose-saline (0.85% NaCl, 0.2% glucose). Micro-pipettes for serial dilution were prepared and weight-calibrated as described by Fildes (1931).

Paper Chromatography of Acid Dyes

R_F values of dyes were obtained from ascending chromatograms on Whatman No. 50 paper, using a benzyl alcohol/ethanol/10N-H_2SO_4 mixture in proportions of 45/25/15 (v/v), and on Whatman No. 1 paper using an *n*-butanol/acetic acid/water mixture in proportions of 40/10/50 (v/v).

Origin of Trypanocides

Butarsen [γ-(*p*-arsenosophenyl)butyric acid monohydrate], tryparsamide (sodium *p*-carbamoylmethylaminophenylarsonate), pentamidine isethionate [1,5-di-(*p*-amidinophenoxy)pentane diisethionate], stilbamidine hydrochloride [di(*p*-amidino)stilbene dihydrochloride], and dimidium bromide (2,7-diamino-10-methyl-9-phenylphenanthridinium bromide) from May and Baker.

Homidium bromide (Ethidium bromide, 2,7-diamino-10-ethyl-9-phenylphenanthridinium bromide) from Boots. Oxophenarsine hydrochloride (Mapharside, hemialcoholate of 3-amino-4-hydroxyphenylarsenoxide hydrochloride) and melarsen (disodium *p*-melaminylphenylarsonate) from Parke, Davis. Surfen [1,3-di(4-amino-2-methylquinol-6-yl)-urea hydrochloride] from Bayer Products. Surfen C [Congasin, di(4-amino-2-methylquinol-6-yl)melamine] from Bayer I.G. Farbenindustrie. Synthalin B (dodecamethylenebisguanidine dihydrochloride) as used by Lourie and Yorke (1937). Decane diamidine (decamethylenebisamidine dihydrochloride) and 3-acetamido-4-carboxyphenylarsenoxide from the late Dr. H. King, National Institute for Medical Research.

Quinapyramine [Antrycide, 4-amino-6-(2-amino-6-methylpyrimidin-4-ylamino)quinaldine-1:1′-dimetho-sulphate], suramin {hexasodium salt of 3″,3″-urylenebis[8-(3-benzamido-4-methylbenzamido)-naphthalene-1,3,5,-trisulphonic acid]}, and para rosaniline [Colour Index No. 676 (1st Edn.); 42500 (2nd Edn.)] from Imperial Chemical Industries. Berenil [di(*p*-amidinophenyl)-N-1,3,-triazene diaceturate trihydrate] from Farbwerke Hoechst. Cinnoline 528 [N^1N^3-di(4-aminocinnol-6-yl)guanidine dimethiodide] as used by Lourie, Morley, Simpson, and Walker (1951). Trypan blue (a) [C.I. No. 477 (1st Edn.); 23850 (2nd Edn.)], azovan blue [tetrasodium 4,4′-di(8-amino-1-hydroxy-5,7-disulphonaphth-2-ylazo)-3,3′-ditolyl], pontamine sky blue 5BX [C.I. No. 520 (1st Edn.); 24400 (2nd Edn.)], trypan red [C.I. No. 438 (1st Edn.); 22850 (2nd Edn.)], congo red [C.I. No. 370 (1st Edn.); 22120 (2nd Edn.)], benzopurpurin 4B [C.I. No. 448 (1st Edn.); 23500 (2nd Edn.)], azo blue [C.I. No. 463 (1st Edn.); 23680 (2nd Edn.)], vital red [C.I. No. 456 (1st Edn.); 23570 (2nd Edn.)], dianil blue 2R [C.I. No. 465 (1st Edn.); 23690 (2nd Edn.)], isamine blue [C.I. No. 710 (1st Edn.); 42700 (2nd Edn.)], and xylene cyanol FF [C.I. No. 715 (1st Edn.); 43535 (2nd Edn.)] from G. T. Gurr, Ltd.

Trypan blue (b), chlorazol fast pink BK [C.I. No. 353 (1st Edn.); 25380 (2nd Edn.)], acriflavine (mixture of 2,8-diaminoacridine dihydrochloride and 2,8-diamino-10-methylacridinium chloride), proflavine (2,8-diaminoacridine) sulphate, and aminacrine (5-aminoacridine) hydrochloride from British Drug Houses.

Results

Table I shows the effective doses of active trypanocides in mice infected with the normal parent strain of *T. rhodesiense*. The following compounds were inactive at the LD10 level: aminacrine, azovan blue, pontamine sky blue 6BX, trypan red, congo red, benzopurpurin 4B, azo blue, vital red, dianil blue 2R, isamine blue, and xylene cyanol FF.

TABLE I

ACTIVITY OF TEST DRUGS AGAINST NORMAL *T. RHODESIENSE*

Intraperitoneal doses in mg./kg. of body weight. The activity of trypan blue (a) was apparent only on very light infections and at very much higher doses than recorded elsewhere (Morgenroth and Freund, 1924; Ormerod, 1952; Schumacher and Schnitzer, 1956).

Trypanocide	ED90	CD90	LD10
Tryparsamide	750	1,000	2,250
Oxophenarsine	0·75	4·0	10
Acriflavine	5·0	—	20
Proflavine	25	—	25
Melarsen	30	70	340
Melarsen oxide	0·125	1·25	5·0
Sodium *p*-melaminyl-phenylstibonate polymer (MSb)	20	30	1,150
MSb 3	0·25	1·25	25
Synthalin B	7·5	—	7·5
Decane diamidine	12·5	—	12·5
Pentamidine	0·65	12·5	50
Stilbamidine	0·65	2·5	50
Berenil	—	5	100
Surfen	50	200	200
Surfen C	50	200	200
Quinapyramine	1·6	—	15
Cinnoline 528	0·75	—	25
Dimidium	5·0	—	50
Homidium	1·25	2·5	12·5
Para rosaniline	12·5	—	12·5
Butarsen	0·75	5·0	12·5
3-Acetamido-4-carboxy-phenylarsenoxide	17·5	—	17·5
Suramin	1·35	5·0	250
Trypan blue (a)	2,000	—	2,000
(b)	62·5	—	250
Pontamine sky blue 5BX	125	—	125

TABLE II

R_F VALUES OF ACID DYES

(1) Benzyl alcohol/ethanol/10N H_2SO_4 (45/25/15, v/v) on Whatman No. 50 (20°). (2) Butanol/acetic acid/H_2O (40/10/50, v/v) on Whatman No. 1 (28°).

Acid Dye	R_F Value		Trypanocidal Action
	(1)	(2)	
Xylene cyanol FF	Approx. 1		Nil
Isamine blue	0·66		,,
Azovan ,,	0·24, 0·63	0·16	,,
Trypan ,,	0·01	0·08	Active
Pontamine sky blue 5BX	0·02	0·04	,,
,, ,, ,, 6BX	0·09	0·12	Nil

Table II shows the R_F values of six sulphonated dyes, of which the last four are closely related in structure. The two trypanocidally active dyes are seen to be the most strongly adsorbed, that is they give the lowest R_F values.

Approximate times taken to produce maximal drug resistance by the "relapse" method (Schnitzer and Grunberg, 1957) were respectively: *atoxyl-resistant*, 5 weeks (Yorke, Murgatroyd, and Hawking, 1932); *butarsen-resistant*, 16 weeks (30 treatments); *melarsen-resistant*, 18 weeks (30 treatments); *acriflavine-resistant*, 56 weeks (76 treatments); *stilbamidine-resistant*, 59 weeks (Fulton and Grant, 1955); *Surfen C-resistant*, 16 weeks (21 treatments); *suramin-resistant*, 36 weeks (originally developed by Hawking (1939) and treated later to maximal resistance levels by Fulton and Yorke (1941)); *pontamine sky blue 5BX-resistant*, 13 weeks (subsequently treated for a further 38 weeks; 80 treatments in all).

No loss of the parabasal body was apparent in any of the fully-developed resistant strains except in the Surfen C-fast strain, where the proportion of trypanosomes with a parabasal body decreased *pari passu* with acquisition of resistance until the loss was complete when the strain became fully resistant.

Table III shows the cross-resistance pattern obtained by treatment of the various strains with the series of drugs and doses listed in Table I.

Discussion

The cross-resistance pattern revealed in Table III conforms in the main to the grouping of trypanocidal drugs according to resistance behaviour which has been established by the work of Ehrlich, Franke, and Roehl (Ehrlich, 1907), Browning (1908), Yorke and Murgatroyd (1930), Yorke, Murgatroyd, and Hawking (1931, 1932), and King and Strangeways (1942). We would emphasize that the results in Table III have been obtained using offshoots of one parent strain only, the Liverpool *T. rhodesiense* strain, and our results are comparable not only among themselves but with those of other workers who have used the same strain, for example, Yorke and Murgatroyd (1930), Yorke *et al.* (1931, 1932), Lourie and Yorke (1938), Hawking (1938), Fulton and Yorke (1941), and Fulton and Grant (1955).

Ehrlich and his collaborators distinguished four main classes of trypanocide: (i) parafuchsin (para rosaniline) and related green, blue, violet and red triphenylmethane dyes (Table III. triphenylmethane dyes group), (ii) benzidine azo dyes (trypan red, trypan blue, and trypan violet

TABLE III

ACTIVITY OF TEST DRUGS AGAINST RESISTANT STRAINS OF *T. RHODESIENSE*

Degrees of resistance: −, normally sensitive; +, fast to ED90 only; ++, fast to CD90 only; +++, fast to LD10. N.D. indicates not done; (a), trypan blue sample (a). The asterisks refer to responses also found *in vitro*.

Drug Group	Drug	Ionization at Blood *p*H	Response of Strain Resistant to							
			Atoxyl	Acriflavine	Melarsen	Stilbamidine	Surfen C	Butarsen	Suramin	Pontamine Sky Blue 5BX
Neutral aromatic arsenicals	Tryparsamide	Feeble	+++	++	+++	+++	−	+++	−	+++
	Oxophenarsine		+++*	+++	+++	+++	−	+++	−	+++
Melaminyl arsenicals and antimonials	Melarsen	Cationic	−*	−	+++	+++	+	−	−	−
	Melarsen oxide		−*	N.D.	+++*	++*	+	−	−	N.D.
	MSb		−*	,,	+++	+++	++	−	−	,,
	MSb3		−*	,,	+++	+++	++	−	−	,,
Acridine derivatives	Acriflavine		+++	+++	+++	+++	+	+++	−	−
	Proflavine		N.D.	+++	+++	+++	N.D.	N.D.	N.D.	−
Diguanidines and diamidines	Synthalin B		−	−	+++	+++	−	−	−	−
	Decane diamidine		−	−	+++	+++	−	−	−	−
	Pentamidine		−	−	+++*	+++*	+++	−	−	−
	Stilbamidine		−	N.D.	+++	+++	+++	−	−	N.D.
	Berenil		N.D.	−	N.D.	+++	N.D.	N.D.	N.D.	−
6-Aminoquinoline and 6-amino-cinnoline derivatives	Surfen		−	−	−	+++	+++	−	−	−
	Surfen C		−	−	−	+++	+++	−	−	−
	Quinapyramine		−	+++	−	−	+++	−	−	−
	Cinnoline 528		−	−	++	+++	N.D.	−	−	−
Phenanthridinium derivatives	Dimidium		−	−	++	+++	+++	−	−	−
	Homidium		N.D.	−	−	+++	N.D.	−	N.D.	−
Triphenylmethane dyes	Para rosaniline		−	−	+++	+++	+++	±	−	−
Carboxylated aromatic arsenicals	Butarsen	Anionic	−	−	−	−	−	+++*	−	−
	3-Acetamido-4-carboxyphenyl-arsenoxide		−*	N.D.	N.D.	−	N.D.	+++	−*	N.D.
Sulphonated naphthylamine derivatives	Suramin		−	−	−	−	−	−	+++	−
	Trypan blue		− (a)	−	N.D.	+	− (a)	− (a)	± (a)	+
	Pontamine sky blue 5BX		−	−	−	++	N.D.	−	−	+++

(Table III, sulphonated naphthylamine derivatives group), (iii) heterocyclic orthoquinonoid dyes of tricyclic anthracene structure containing one or two heteroatoms in the *meso* position (for example, derivatives of pyronine, acridine, selenopyronine, phenoxazine, phenothiazine, and phenoselenazine) (Table III, acridine derivatives group), and (iv) aromatic arsenicals of the atoxyl type (Table III, neutral aromatic arsenicals group). They showed that strains resistant to (i) or (ii) were sensitive to members of all the other groups. Mutual cross-resistance occurred between groups (iii) and (iv), but strains resistant to either group were still sensitive to groups (i) and (ii).

Yorke and his colleagues showed that resistance to aromatic arsenicals was not towards arsenic itself but to the non-arsenical part of the molecule, and King and Strangeways (1942) were able to subdivide aromatic arsenicals into three groups: (*a*) having hydrophilic substituents feebly ionized at blood *p*H (Table III, neutral aromatic arsenicals group), (*b*) lacking hydrophilic or having lipophilic substituents, and (*c*) having substituents which ionize as anions at blood *p*H (Table III, carboxylated aromatic arsenicals group). Trypanosomes resistant to (*a*) (atoxyl-type) were sensitive to (*b*) and (*c*), but strains resistant to (*c*) (butarsen-type), although sensitive to (*b*), were resistant also to (*a*).

To these three sub-classes, of which (*b*) and (*c*) represent Ehrlich's "avid" arsenicals (those active against strains resistant to the atoxyl-type), we can add a fourth (*d*) comprising aromatic arsenicals with a melaminyl substituent ionizing as the cation at blood *p*H. Strains resistant to (*a*) and (*c*) are sensitive to (*d*) (Williamson and Lourie, 1948); this is also an "avid" type of arsenical. Strains resistant to (*d*) are resistant to (*a*) but not to (*c*) (Table III). Schnitzer and Grunberg (1957) have indicated that strains resistant to these "avid" arsenicals seem invariably to show cross-resistance to acriflavine-type and to para rosaniline-type dyes, although atoxyl-resistant strains which are resistant also to acriflavine-type dyes are sensitive to para rosaniline. The connexion between cross-resistance behaviour and

selective interference with trypanocidal action is discussed by Williamson (1959). Schnitzer and Grunberg (1957) have compiled evidence which seems to "indicate that *p*-rosaniline occupies a key position in the pattern of cross-resistance of the arsenicals."

A number of other groups of metal-free trypanocides can also be distinguished on the basis of cross-resistance behaviour: (i) a group of symmetrical sulphonated naphthylamine derivatives which includes suramin and trypanocidal benzidine azo dyes, (ii) the diamidines, and (iii) a group of 6-aminoquinoline derivatives and related compounds. All three groups are active against trypanosomes resistant to aromatic arsenicals of the atoxyl type.

In group (i), suramin-resistance (Morgenroth and Freund, 1924) is difficult to induce and is highly specific; its only known cross-resistance is to the benzidine azo dyes trypan red and trypan blue; strains resistant to trypan blue are resistant to suramin (Leupold, 1924).

In group (ii), diamidine resistance was reported by Lourie and Yorke (1938), who showed that strains resistant to Synthalin (an aliphatic diguanidine) and to undecane diamidine showed cross-resistance to other aliphatic diguanidines and to aliphatic and aromatic diamidines, but were sensitive to suramin and to aromatic arsenicals. A strain made resistant to the aromatic diamidine stilbamidine (Fulton and Grant, 1955) is, however, cross-resistant to a much wider variety of trypanocides (notably to the melaminyl group), and is sensitive only to suramin, quinapyramine, and carboxylated aromatic arsenicals (Williamson and Rollo, 1952) (Table III). The new diamidine, berenil, like the other members of the group, is active against the acriflavine- and pontamine sky blue 5BX-fast strain, and, as Hawking (1958) has also shown, is inactive against a stilbamidine-fast strain.

The group of trypanocidal derivatives of 6-aminoquinoline and 6-aminocinnoline (group iii) is not strictly homogeneous chemically, but there are several aspects of resistance behaviour in this group which are of interest, despite the incomplete nature of the evidence. Surfen and Surfen C are symmetrical bisquinoline compounds, with a 6,6′ linkage through urea in the former and through melamine in the latter; cinnoline 528 has a similar symmetrical biscinnolinium structure linked in the 6,6′ positions by a guanidine group. These three drugs are uniformly active against trypanosomes resistant to neutral and anionic arsenicals and to acriflavine, and also to suramin and acid dyes, but are inactive against strains resistant to stilbamidine. Cinnoline 528, but not Surfen or Surfen C, is inactive against a melarsen-resistant strain. A Surfen C-fast strain showed no cross-resistance to neutral and anionic arsenicals, to aliphatic amidines and guanidines, or to suramin and acid dyes; it is, however, cross-resistant to melaminyl arsenicals and antimonials, aromatic diamidines, quinapyramine, dimidium, and para rosaniline.

Although we can discern a pattern in the resistance behaviour of the melaminyl arsenicals, the diamidines, the Surfens and cinnoline 528 in the common presence of amidine type $=N-\overset{|}{C}=N-$ linkages, capable of tautomerism and thus peculiarly prone to hydrogen-bond formation, it is difficult to construct an appropriate receptor structure, modification or loss of which would account for all the observed facts. On this basis why should the Surfen C-fast strain be able to differentiate between aliphatic and aromatic amidines while the melarsen-fast strain does not? Is this another aspect of the sensitivity to neutral aromatic arsenicals which the Surfen C-fast and aliphatic amidine-fast strains display, unlike strains fast to other basic metal-free trypanocides such as acriflavine, stilbamidine, and styryl-6-amino-quinolines (Browning, Cohen, Ellingworth, and Gulbransen, 1929)?

Quinapyramine is an asymmetrical 6-amino-quinoline derivative, and the cross-resistance shown to it by the Surfen C-fast strain can be referred to the 4,6-diaminoquinaldine moiety which is common to both compounds. Quinapyramine is active against all the other resistant strains in Table III except the acriflavine-fast strain. It is also inactive against a strain of *T. congolense* resistant to cinnoline 528, but an analogous quinapyramine-fast *T. congolense* is sensitive to cinnoline 528 (Williamson and Lourie, unpublished observations). A quinapyramine-fast strain of *T. equiperdum* (Ormerod, 1952) was resistant to the phenanthridinium drug dimidium, to acriflavine and to stilbamidine, but was sensitive to suramin, trypan blue, and *p*-hydroxy-phenylarsenoxide.

Cross-resistance between quinapyramine and phenanthridinium compounds is of considerable importance in animal trypanosomiasis in the field, but available evidence from this source is often contradictory and difficult to establish. This situation, coupled with the difficulty of producing experimental phenanthridinium resistance in laboratory trypanosome strains, does not facilitate the formulation of rules for the resistance behaviour

of these two important drug groups. Such indications as there are point to a non-reciprocal cross-resistance between quinapyramine and dimidium; strains resistant to quinapyramine are cross-resistant to dimidium but not conversely (Wilson, 1949; Macaulay and Shaw, 1950; Robson and Wilde, 1954; Evans, 1956). Dimidium and homidium, which differs from dimidium only in having an ethyl- instead of a methyl-substituted quaternary nitrogen atom, have similar patterns of activity against all the resistant strains tested except the melarsen-fast strain.

We have attempted to extend the evidence for an ionic basis of the trypanocidal action and resistance behaviour of acidic and basic drugs (King and Strangeways, 1942; Schueler, 1947) by using as wide a variety of anionic trypanocides as possible. Unfortunately, of the series of acid dyes tested, several of which had been reported as trypanocidal, only two, trypan blue and pontamine sky blue 5BX, were active against our parent *T. rhodesiense* strain. Because of the relative inactivity of a sample of trypan blue used in the early part of this work, evidence of cross-resistance to trypan blue in some instances (Table III) is not so clear-cut as could be desired. However, within this group of metal-free anionic trypanocides, we have found several features of novel interest. Of the six sulphonated dyes listed in Table II, four are closely related chemically, trypan blue, azovan blue, pontamine sky blue 5BX, and pontamine sky blue 6BX [C.I. No. 518 (1st Edn.); 24410 (2nd Edn.)]. Trypan blue and azovan blue, having a central dimethylbenzidine moiety, differ only in the distribution of sulphonic acid substituents; the former is the bis-3,6-disulphonate, and the latter the bis-2,4-disulphonate. Pontamine sky blue 5BX and pontamine sky blue 6BX form a similar pair, having a central dimethoxybenzidine moiety, and again differ only in that the former is the bis-3,6-disulphonate and the latter the bis-2,4-disulphonate. In both these pairs, only the bis-3,6-disulphonate-substituted dye is trypanocidal; it is also the member which is most strongly adsorbed on cellulose (Table II), and in this respect recalls the relationship between trypanocidal action and cotton substantivity noted by Quastel (1931) in series of aminobenzoyl-substituted derivatives of naphthylamine disulphonates related to suramin. Sulphonated dyes generally are taken up readily by wool, which is a protein with basic groups, but the uptake of these dyes by cellulose is structurally much more limited and specific. The size, shape, and composition of the non-sulphonated part of the dye play a large part in the degree of its ultimate adsorption. In the case of the 2,4- and 3,6-disulphonated dyes considered here, there is the possibility of a solubility difference between the two groups which would be likely to affect their adsorbability on both protein and cellulose structures (W. Bradley, personal communication). The enhancement of cotton substantivity in azo dyes by the *s*-triazine nucleus (Bradley, 1958) which occurs in the melamine trypanocides suggests a further link between trypanocidal action and affinity for cellulose.

The cross-resistance pattern (Table III) of the trypanocidal sulphonated naphthylamine derivatives shows that although cross-resistance exists between suramin and trypan blue, and to a small extent between trypan blue and pontamine sky blue 5BX, there is no cross-resistance between suramin and pontamine sky blue 5BX, although the latter differs from trypan blue only in having dimethoxy instead of dimethyl substituents on the central benzidine moiety. A further unexpected feature in this group is that the stilbamidine-resistant strain, although normally sensitive to suramin, is cross-resistant to some degree to both the benzidine azo dyes, trypan blue and pontamine sky blue 5BX. Later (Williamson, 1959b) it will be shown that the stilbamidine-resistant strain is resistant *in vitro* also to another acid dye, indigo carmine, and that the degree of resistance, as with a number of other ionic trypanocides, is a function of the *p*H of the culture medium. Uptake in these instances is determined by the degree of ionization of a cellular receptor.

One of the more plausible current theories of the resistance mechanism in trypanosomes (Schueler, 1947) implies that the ionic properties of a drug molecule control to a large extent the subsequent cross-resistance behaviour of a strain made resistant to it. For example, the strain made resistant to the atoxyl-tryparsamide-oxophenarsine-type of arsenical, in which the substituents are hydrophilic but ionize only feebly or not at all, is normally sensitive to trypanocides with ionizable substituents (anionic or cationic). This scheme is, however, an oversimplification, as it fails to explain (Table III) why (i) the cationic arsenic-free Surfen C gives rise to a resistant strain which is sensitive to arsenicals of the atoxyl type and to aliphatic diamidine-type drugs, but resistant to quinapyramine, while the similarly cationic arsenic-free stilbamidine produces a strain resistant to the two former groups but sensitive to quinapyramine; (ii) the cationic arsenic-free acriflavine produces resistance to atoxyl-type arsenicals and

quinapyramine only; (iii) the three strains made resistant to the anionic drugs butarsen, suramin, and pontamine sky blue 5BX are not cross-resistant to each other; and (iv) of four strains made resistant with cationic drugs, one, the stilbamidine-fast, is cross-resistant to anionic benzidine azo trypanocides. Other inconsistencies in the theory have been noted by Wagner, Pedal, and Schöneberger (1954).

The resistance produced by stilbamidine apparently involves a more fundamental change in the drug "receptors" than is implied by the suggestion of Schueler (1947) of a shift in the isoelectric point of protein in resistant strains. If this mechanism operated in the stilbamidine-fast strain, the decrease in net negative charge of the receptor structure, as manifested by resistance to cationic drugs, should be accompanied by an increased net positive charge tending to produce an increased sensitivity to anionic trypanocides, instead of the resistance which is actually found. In this respect the strain differs from the quinapyramine-resistant strain of *T. equiperdum* described by Ormerod (1952), which, though resistant also to stilbamidine, was sensitive, or possibly hypersensitive, to the anionic trypan blue and suramin.

From Table III it seems that a number of drug adsorption sites are likely to be common or adjacent to each other. The largest "receptor area" would appear to be anionic in nature, and may well consist of free phosphoric acid groups of nucleic acids, because cationic trypanocides such as aromatic diamidines, acriflavine, triphenylmethane dyes, and phenanthridinium drugs do in fact combine with nucleic acid *in vitro*.

The difference in action of aliphatic and aromatic diamidines on the metabolism of *T. evansi* (Marshall, 1948) may be reflected in the marked difference in resistance behaviour between the stilbamidine-fast and the Synthalin- and undecane diamidine-fast strains described by Lourie and Yorke (1938). Unlike the first of these strains, the two last were both normally sensitive to an atoxyl-type arsenical, dichlorophenarsine (halarsol). Further, the Surfen C-fast strain, which is fully resistant to pentamidine and stilbamidine, is sensitive to the aliphatic compounds Synthalin and decane diamidine, and also to aromatic arsenicals of the atoxyl type.

Changes in cell metabolism whereby drug-sensitive enzyme reactions are modified, by-passed or eliminated may well be linked to changes in structures associated with drug uptake. Some changes in the metabolism of *T. rhodesiense* induced by resistance to melarsen have been demonstrated (Williamson, 1953), but nothing in this work or in the work of others (Fulton and Christophers, 1938; Harvey, 1948, 1949; von Brand, Tobie, Mehlman, and Weinbach, 1953; Tobie and von Brand, 1954) has yet revealed any far-reaching differences in intermediary metabolism to correspond to the relatively enormous losses in drug sensitivity found in resistant trypanosomes.

One of the oldest and still one of the most refractory problems in trypanocidal drug resistance is the nature of the cross-resistance between acriflavine and atoxyl-type aromatic arsenicals. Thiol compounds can reverse the trypanocidal action of the arsenicals but not of acriflavine, and further evidence of a separation in the function of a thiol-type arsenical "receptor" is provided by the Surfen C-fast strain which is resistant to acriflavine but not to atoxyl-type arsenicals. Possibly the action of acriflavine involves oxidation-reduction changes in the thiol receptor to which the arsenic is ultimately linked by covalent bonds. Albert (1951) has indicated that antibacterial acridines can be divided into two classes, (i) mainly aminacrine and its derivatives, which resist oxidation by light and air and iron-catalysed hydrogen peroxide, and (ii) mainly 2-aminoacridine derivatives, which are susceptible to these oxidizing agents. Resistance in bacteria develops only with this latter class. It may be relevant that the active antibacterial substance aminacrine, unlike proflavine and acriflavine which belong to class (ii), has no trypanocidal action.

There is no detectable difference in the thiol content of normal and arsenical-resistant trypanosomes (Harvey, 1948), but Hawking (1938) has shown that resistant trypanosomes can be killed by a much smaller amount of intracellular arsenic or acriflavine than normal trypanosomes. This suggests that in the resistant parasite a proportion of metabolically active thiol groups has been masked by some form of structural rearrangement; a masking effect of this sort would not normally be detectable by ordinary chemical methods of thiol analysis.

Results of selective interference experiments (Williamson, 1959a) confirm the observations presented here, which suggest that stereospecific structural changes associated with initial drug uptake occur in resistant trypanosomes. The complexity of these changes appears to be of similar degree, if not of similar nature, to that of antigen-antibody interaction.

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DRUG RESISTANCE IN TRYPANOSOMES; SELECTIVE INTERFERENCE WITH TRYPANOCIDAL ACTION

BY

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Selective reversal of the trypanocidal action of carboxylated arsenicals by *p*-aminobenzoic acid and of melaminyl arsenicals and diamidines by melamine has been demonstrated *in vivo* and *in vitro*. The structural specificity of these reversal phenomena is high, and suggests preferential adsorption of the antagonist during a reversible primary drug fixation stage. Thiols antagonize neutral, carboxylated and melaminyl aromatic arsenicals equally, but not diamidines; *p*-aminobenzoic acid antagonism is specific for carboxylated arsenicals, and melamine antagonizes only the melaminyl arsenicals and the diamidines. These reversals reflect the pattern of cross-resistance behaviour and suggest that cellular structures associated with a reversible stereospecific drug adsorption phase are modified during the development of resistance.

Antagonism of the chemotherapeutic activity of one substance by another was originally termed "therapeutic interference" by Browning and Gulbransen (1922), who made experiments on the reversal of the trypanocidal action of acriflavine by parafuchsin. This and later examples of the effect have been reviewed in detail by Schnitzer and Grunberg (1957) and by Albert (1949, 1951).

The present work is based on the observation in trypanosome infections of antagonism of butarsen by *p*-aminobenzoic acid (Williamson and Lourie, 1946), antagonism of melarsen oxide by Surfen C (Williamson and Lourie, 1948), and antagonism of diamidines and melaminyl arsenicals by melamine (Williamson, 1954). The more extended experiments now reported indicate a high degree of structural specificity in these reversal phenomena, many of which are reproducible on isolated parasites *in vitro*.

METHODS

Trypanosome Strain.—The strain of *Trypanosoma rhodesiense* used throughout these experiments was isolated from man in 1923 (Yorke, Adams, and Murgatroyd, 1929), and subsequently maintained by blood passage in mice.

Estimation of Interference.—Mice weighing 18 to 25 g. were injected intraperitoneally with a suspension of 20,000 trypanosomes, and about 43 hr. later microscopical examination of fresh tail blood showed an average count of 1 to 10 parasites/field (1/6 in. objective; ×10 ocular).

Four or five mice were used for each test group, and each mouse was injected intraperitoneally with a neutral solution of the antagonist (0.5 ml./20 g. body weight), followed 2 to 3 min. later by an intraperitoneal ED90 dose (0.5 ml./20 g.) of the trypanocide. In each experiment, four or five infected mice were treated with the trypanocide only as controls. LD10 doses of test antagonists were also determined where necessary. ED90 and LD10 are defined by Williamson and Rollo (1959).

After treatment, blood examinations were made at intervals of 30 min. or 1 hr., and, if necessary, at intervals of 24, 48, and 72 hr. The criterion of negativity throughout was the absence of trypanosomes in thirty microscope fields selected at random.

For analysis of the extent of antagonism of a fixed dose of butarsen by varying doses of *p*-aminobenzoic acid, mice were infected as above, and, 48 hr. later, blood films were made and stained. Immediately afterwards, batches of five mice were given intraperitoneal doses (0.5 ml./20 g.) of sodium *p*-aminobenzoate followed 2 to 3 min. later by the intraperitoneal injection (0.5 ml./20 g.) of a solution of the sodium salt of butarsen. A batch of five mice treated with the drug alone served as controls. Times of treatment of each batch were noted, and, exactly 1 hr. later, stained blood films were made from the tail blood of the mice in each batch. Differential parasite counts on the stained films gave infection concentrations in terms of parasites/4,000 erythrocytes. Mean parasite concentrations for each batch of five mice were calculated and the standard error of these mean values estimated. The proportion (x) of trypanosomes killed at each treatment level was obtained from the ratio $x=(A-B)/A$, where $A=$

mean initial count and B=mean count after treatment. The amount of inhibition (I) of the trypanocidal action of the drug was estimated from the ratio: $I = [x_D - x_{CT}]/x_D$, where x_D is the ratio x for the arsenical alone, and x_{CT} the ratio for the combined treatment by arsenical and antagonist. In batches of mice where the concentration of antagonist was sufficient to produce permanent inhibition, this ratio (x) was found to be greater than 1 by a factor representing multiplication of the trypanosome population during the hour elapsing between treatment and examination. This factor was found to have a mean value of 1.25 for the highest concentration of *p*-aminobenzoate at which antagonistic activity was maximal. The proportionate inhibitions at lower concentrations were divided by 1.25 to give the corresponding values representing a stationary population. The percentage inhibition of the arsenical was assumed to be a measure of the amount of *p*-aminobenzoic acid adsorbed on the site of action of the drug.

The "interference activity" of compounds tested against butarsen is expressed in terms of activity/mole, taking the activity of *p*-aminobenzoic acid as 100, and was calculated in the following way. The average time of persistence (in hr.) of parasites in the peripheral blood of mice subjected to combined treatment, in excess of the persistence time of parasites in mice treated with butarsen alone, was divided by the molar concentration of the interfering compound, and this quotient was expressed as a percentage of the maximum quotient, calculated similarly, for *p*-aminobenzoic acid. The time of persistence with each compound was measured in two ways: (A) for persistence of parasites in undiminished numbers, and (B) for persistence of parasites in diminished numbers. Activities/mole were calculated using (A) and (B), and, taking the corresponding values for *p*-aminobenzoic acid (1,536 and 3,072 respectively) as 100, the relative activity/mole of each compound tested was found approximately to correspond for both calculation procedures.

In batches of four or five mice, the persistence times were generally found to be uniform to within 1 hr., particularly in the case of (A), and the control mice used in each experiment and treated with the ED90 of butarsen showed a uniform disappearance of parasites within 0.5 to 1.0 hr. after treatment.

The "effective interfering dose" (the minimum dose necessary to produce maximum interference) was determined for each of the compounds related to *p*-aminobenzoic acid.

Throughout, the molar concentrations referred to are those of the test solutions administered intraperitoneally in doses of 0.5 ml./20 g.

Trypanocidal Action in Vitro.—This was measured by the technique of Yorke and Murgatroyd (1930) in the manner described by Williamson and Rollo (1959).

Organic Preparations.—Sodium pyruvate: prepared by the method of Robertson (1942). p-*Aminocinnamic acid*: from ethyl *p*-nitrocinnamate, hydrolysed to *p*-nitrocinnamic acid, thence reduced by the method of Schofield and Simpson (1945), to give yellow-brown crystals of *p*-aminocinnamic acid. M.P. (dec.)=176°, M.P. (lit.)=175–6°. Found: C=66.53%; H=5.81%; N=10.3%. Calc. for $C_9H_9O_2N$: C=66.24%; H=5.56%; N=8.59%. p-*Aminomethylbenzoic acid*: by the method of Blicke and Lilienfeld (1943). The acid, M.P.=230°, was insoluble in ether, chloroform, acetone, and benzene (Günther, 1890), and was used without further purification. p-*Aminobenzamide*: *p*-nitrobenzamide was prepared from *p*-nitrobenzoyl chloride by the method of Reichenbach and Beilstein (1864), and was then reduced by either (i) catalytic hydrogenation or (ii) stannous chloride. Method (i) gave, on recrystallization from water, yellow-brown flakes of *p*-aminobenzamide. M.P.=180° (M.P. (lit.)=180°, 182.9°). Found: C=61.51%; H=5.75%; N=21.0%. Calc. for $C_7H_8ON_2$: C=61.75%; H=5.92%; N=20.58%. Method (ii) gave a similar product, M.P.=182.5°.

Determination of p*K Values.*—p*K* values of the acidic groups (and in some cases of the basic groups also) of *p*-substituted aromatic acids and phenylarsonates were determined at 19° to 20° by potentiometric titration with the glass electrode of the sodium salts, either in water or in 50% (v/v) aqueous methanol. The method has been adopted for relatively insoluble members of compound types of therapeutic interest such as the sulphonamides (Bell and Roblin, 1942) and the aminoacridines (Albert and Goldacre, 1943).

All pK_a values were corrected for variation in ionic strength, salt hydrolysis, Na^+ error, and the effect of alcohol.

Surface Tension Determination.—Correlation of antibacterial action and surface activity is largely dependent on measurements of surface tension made over a range of concentrations in the appropriate culture medium (Stacey and Webb, 1947). The measurements recorded here were made in solutions of rabbit serum and physiological saline, using the dynamic maximum bubble-pressure method of Sugden (1922, 1924). This technique overcomes a number of objections inherent in static methods (Neurath and Bull, 1938; Gaddum, 1931), and its application to measurements in serum is discussed in detail elsewhere (Williamson, 1959). The apparatus was calibrated and checked against distilled water, absolute ethanol (redistilled), acetone (A.R.), and benzene (A.R.). The temperature throughout was controlled at 20°.

In all experiments the given value for the surface tension is the mean of at least three determinations, each determination involving four scale readings. The value of H for the saline and serum/saline solvents was about 12.0 cm., corresponding to about 72 dynes/cm., and the means are accurate to ±0.3 dyne/cm.

Cell-free rabbit serum was obtained by centrifuging blood obtained by cardiac puncture, which had been

left for an hour at 37° to clot. A well-defined minimum in the surface tension of solutions of rabbit serum in 0.85% aqueous NaCl occurred at approximately 15% (v/v) serum; above 40% (v/v) serum the surface tension became constant and equal to that of undiluted serum. A concentration of 1% (v/v) serum gave a surface tension close to that of undiluted serum; the measurements recorded here were therefore made in a solution of this strength, in which protein molecular association, considered to be responsible for the observed surface tension minimum, would be unlikely to operate.

As far as possible all test solutions were adjusted to *p*H 7.0; the *p*H of solutions in serum-saline mixtures was determined with the glass electrode. A surface tension/*p*H curve was constructed for a series of 1% serum solutions in buffered saline (Williamson, 1959), in order to correct the surface activity in unbuffered test solutions, in the following way. The surface tension of a sample of 1% serum/saline dilution used for the preparation of a batch of test solutions was determined and compared with the value at the corresponding *p*H on the surface tension/*p*H curve, and a correction factor (σ curve value/σ observed value) obtained. The surface tension of the test solution was multiplied by this factor, and the corrected value subtracted from the value of the serum/saline solvent at the corresponding *p*H on the surface tension/*p*H curve. The standard value of 71.9 dynes/cm. for a 1% solution of rabbit serum in 0.85% aqueous NaCl at *p*H 7.0 was taken as a comparative basis for the results.

The above correction procedure is only valid in the *p*H range 6.0 to 10.0, in which surface tension measurements are reproducible, and is based on the assumption that within this range the surface tension/*p*H curves for the solution and the solvent are parallel.

Results

p-Aminobenzoic Acid/Butarsen Interference

p-Aminobenzoic acid/butarsen interference could be demonstrated *in vitro* only under limited conditions (Williamson and Lourie, 1946), and therefore analysis of the quantitative relations involved had to be based on mouse experiments which could be less rigidly controlled. Although *p*-aminobenzoic acid produced permanent inhibition of an ED90 dose of butarsen (0.75 mg./kg. body weight), an increase of 0.25 mg./kg. of the arsenical was sufficient to reduce the period of inhibition to only 1 to 2 hr. Analysis of the effect of varying doses of the arsenical combined with a constant dose of *p*-aminobenzoic acid was thus impossible. The antagonistic effects of varying doses of *p*-aminobenzoic acid on the ED90 of butarsen are shown in Table I.

A curve was drawn putting percentage inhibition of the trypanocidal action of 0.015 mg./20 g. mouse body weight of the arsenical solution as the ordinate, and *p*-aminobenzoic acid concentration as the abscissa. This curve seemed to conform to the typical rectangular hyperbola associated with an adsorption process. By putting Gaddum's equation (Gaddum, 1943) into the form of the Langmuir adsorption isotherm: $C_A/b = k + C_A$ (where b represents % inhibition of the arsenical and C_A is the concentration of *p*-aminobenzoic acid, the value of the intercept constant was obtained, and a theoretical curve plotted for comparison with the experimental curve (Fig. 1).

Although the experimental method does not allow rigid control, and the results cannot be considered conclusive, they suggest the possibility that a preferential adsorption of *p*-aminobenzoic acid may be involved in the interference process.

TABLE I

EFFECT OF *p*-AMINOBENZOIC ACID CONCENTRATION ON THE INHIBITION OF THE TRYPANOCIDAL ACTION OF BUTARSEN ON STANDARDIZED *T. RHODESIENSE* INFECTIONS OF MICE

Butarsen dose, 0·5 ml./20 g. of $1{\cdot}18 \times 10^{-4}$ M solution. *p*-Aminobenzoic acid was injected intraperitoneally in a volume of 0·5 ml. The standard error of the mean is given in brackets.

p-Aminobenzoic Acid (M)	Trypanosomes/4,000 R.B.C.		% Inhibition for Stationary Population
	Before Treatment (A)	1 Hr. after Treatment (B)	
1·00	70 (±32)	81 (±42)	96
0·50	182 (±47)	223 (±29)	101
0·125	186 (±17)	246 (±32)	111
0·063	182 (±11)	219 (±47)	100
0·031	164 (±22)	198 (±28)	100
—	255 (±66)	43 (±12)	0
0·016	145 (±16)	113 (±16)	62
0·008	137 (±39)	82 (±35)	46
—	62 (±15)	3 (±2)	0
0·021	335 (±60)	338 (±70)	77
0·004	477 (±88)	346 (±73)	−5
—	502 (±51)	371 (±68)	0

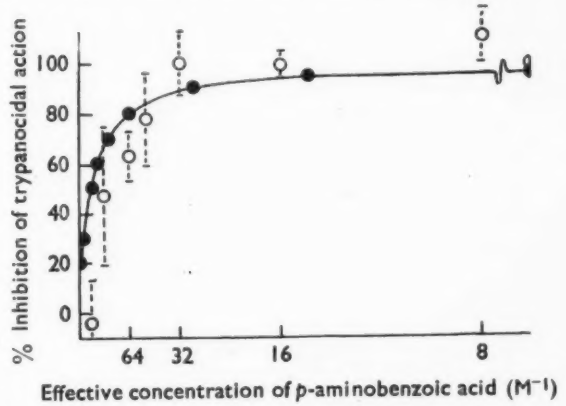

FIG. 1.—Inhibition of trypanocidal action of butarsen by *p*-aminobenzoic acid in mice infected with *T. rhodesiense*, 1 hr. after combined treatment. ●, theoretical value on basis of adsorption isotherm; O, experimental value, with 2× standard error represented by vertical broken line.

As indicated below, this result may be an alternative expression of a normal probability distribution of a drug-susceptibility factor among the large heterogeneous population of parasites in the blood, rather than of receptor sites on a homogeneous parasite surface. The minimum concentration of *p*-aminobenzoic acid required for complete inhibition is 3×10^{-2} M, a molar inhibition ratio of the order of 300:1.

Experimental confirmation that interference takes place in the immediate neighbourhood of the trypanosome was obtained by demonstration of *p*-aminobenzoic acid/butarsen interference when the substances were administered by separate routes, the former subcutaneously and the latter intraperitoneally. This experiment excludes the possibility of extracellular chemical interaction between the drug and its antagonist. Complete and permanent inhibition of the trypanocidal action of butarsen by the prior injection of *p*-aminobenzoate was observed at spacing-intervals ranging from about 30 sec. to 1 hr.

Taking a level of significance for activity/mole as 0.4 (the value for glutathione) for parasite persistence times (A) or (B), no significant interference with the action of butarsen was shown by injection of the following: normal and hypertonic saline, isotonic phosphate buffers (*p*H 4, 5, 6, 7, 8, 9), phosphate-citrate buffers (*p*H 4, 5), borate-succinate buffer (*p*H 5), phthalate buffer (*p*H 5), HCl-saline (*p*H 5), glucose (25% in water), or by LD10 injections of urea, casein hydrolysate, peptone, uracil, xanthine, lactate, pyruvate, succinate, malonate, toluidine blue, ascorbic acid, glutamic acid, aspartic acid, tyrosine, histidine, arginine, glycine, β-aminobutyric acid, methanol, ethanol, mercuric chloride, formaldehyde, phenylguanidine, naphthalene - 2,6 - diamidine, para rosaniline, pentamidine, phenidium (phenanthridinium 897), dimidium, melamine, *o*-nitrobenzoic acid, folic acid, nicotinic acid, and phenylacetic acid.

Table II shows the interference activity against butarsen of compounds related to *p*-aminobenzoic acid and of similar molecular size.

p-Aminobenzoic acid was the only active antagonist which produced complete and permanent inhibition at low concentrations. The structural specificity of the interference effect was high, because isomers of *p*-aminobenzoic acid were inactive, and homologues showed greatly reduced activity. Removal or replacement of either the amino or carboxyl groups in the *p*-aminobenzoic acid molecule also abolished or reduced activity.

TABLE II

INTERFERENCE ACTIVITY AGAINST BUTARSEN OF COMPOUNDS RELATED TO *p*-AMINOBENZOIC ACID

Where the effective interfering dose differs from the LD10, the latter is shown in brackets.

Test Substance	Class	Activity/Mole (*p*-Aminobenzoic Acid = 100) (A)	Activity/Mole (*p*-Aminobenzoic Acid = 100) (B)	Effective Interfering Dose (M^{-1})
p-Aminobenzoic acid	Homologues of *p*-aminobenzoic acid	100	100	32 (0·69)
p-Aminophenylacetic acid		25·0–40·0	25·0	16 (1)
β-(*p*-Aminophenyl)-propionic acid		0·74	1·4	2
γ-(*p*-Aminophenyl)-butyric acid		25–63	100	16 (2)
p-Aminobenzoylglycine		25–50	100	8 (4)
p-Aminocinnamic acid		0·04	0·08	1·41
o-Aminobenzoic acid	Isomers of *p*-aminobenzoic acid	0·00	0·01	1
m-Aminobenzoic acid		0·07	0·06	1
Aniline	Carboxyl group replaced	7·0–10·0	4·1–5·0	4
p-Aminobenzamide		0·33–1·73	0·95–1·65	2
Ethyl *p*-aminobenzoate		4·50	3·65	32
p-Aminobenzenesulphonic acid		0·00	0·00	0·5
Sulphanilamide		6·05–8·0	7·70	8
Sulphadiazine		0·00	0·00	10
p-Aminophenol		0·00	0·00	8
p-Aminophenylarsonate		3·52	2·34	36
p-Toluidine		0·52	0·78	16
p-Phenylene diamine		0·00	0·00	40
Benzidine		0·18	0·52	16
Benzoic acid	Amino group replaced	0·11	0·10	2
p-Acetamidobenzoic acid		0·02	0·02	1
p-Hydroxybenzoic acid		0·20	0·13	2
p-Toluic acid		0·17	0·24	2
p-Nitrobenzoic acid		0·00	0·00	2
p-Hydroxyaminobenzoic acid		0·26	0·25	4
p-Aminomethylbenzoic acid		1·64	1·59	2
β-Indolylalanine	Miscellaneous	>0·23	0·55	10 (20)
Phenylacetic acid		0·13	0·12	2
β-Phenylpropionic acid		2·76	1·55	4
Benzoylglycine		25	20·4	8
N-Phenylglycine		1·25	3·45–5·7	8
Surfen		20–49	5·2–12·7	55

The pK_a values of ionizing groups of active interfering agents and of a number of related trypanocides are given in Tables III, IV and V.

The remaining group of substances which showed significant interference activity against butarsen was a series of surface-active compounds of varying type (Table VI). In some instances, the interference activity on a molar basis was greater than that of *p*-aminobenzoate, but complete and permanent inhibition was never observed.

The molar interference activity (persistence method A) was determined when butarsen was

TABLE III

pK_a VALUES IN WATER

The asterisk refers to results interfered with by precipitation of free acid; †, estimated by extrapolation; ‡, as anhydrous disodium salt.

Compound	Temp. (°)	pK_a (Carboxyl or Arsonic Acid)		Temp. (°)	pK_a (Basic Group)	
		Uncorrected	Corrected		Uncorrected	Corrected
p-Aminobenzoic acid	20	4·85	4·81	20	2·80	2·51
p-Aminophenylacetic acid	19	5·30	5·26	19	3·68	3·54
β-(*p*-Aminophenyl)propionic acid	19	5·48	5·44	19	*ca.* 4·1†	
γ-(*p*-Aminophenyl)butyric acid	20	5·52	5·48	20	*ca.* 4·3†	
p-Aminocinnamic acid	19	4·98	4·94	19	*ca.* 3·7†	
p-Hydroxyaminobenzoic acid	19	*ca.* 5·8*				
Melarsen‡	20	5·20	5·16	19	7·70	7·55
Butarsen	20	4·70	4·66			

TABLE IV

pK_a VALUES IN 50% (V/V) AQUEOUS METHANOL

Values marked with an asterisk are uncorrected; †, indicates sodium *p*-acetamidophenylarsonate; ‡, sodium 4-acetamido-3-hydroxyphenylarsonate.

Compound	Temp. (°)	pK_a (Arsonic or Carboxylic Acid Group)		Estimated pK_a (H_2O)
		Uncorrected	Corrected	
Atoxyl	19	4·85	4·77	3·97
Arsacetin†	19	4·30	4·22	3·42
Orsanine‡	19	4·55	4·47	3·67
Tryparsamide	19	4·90	4·82	4·02
Melarsen	19	5·85	5·77	4·97
Butarsen	19	5·55	5·47	4·67
p-Aminobenzoic acid	19	pK_a (NH_3^+) = 2·72*		
		pK_a (carboxyl) = 5·80*		
p-Aminobenzamide	19	pK_a (NH_3^+) = 2·90*		

TABLE V

pK_a VALUES OF COMPOUNDS RELATED TO *p*-AMINOBENZOIC ACID

References for pK values quoted in this Table and in the text are taken from: Kuhn and Wassermann (1928), Dippy (1939), Landolt-Börnstein (1931, 1936), International Critical Tables (1929), Bell and Roblin (1942), Cohn and Edsall (1943), Pressman and Brown (1943), Albert and Goldacre (1942), Goldacre (1944). Values marked with an asterisk are those determined experimentally (see Tables III and IV).

Acid	pK_a (Carboxyl) of Acid with *p*-Substituent		
	None	–As=O	$-NH_2$
Benzoic	4·20	4·0	4·92, 4·81*
Phenylacetic	4·30	4·35	5·20, 5·26*
β-Phenylpropionic	4·66	4·7	5·42, 5·44*
γ-Phenylbutyric	—	4·9, 4·66*	5·48

given subcutaneously and the surface-active agent intraperitoneally. Three representative compounds (i) cetyltrimethylammonium bromide, (ii) octadecylamine, and (iii) sodium tauroglycocholate gave respectively the values (*p*-aminobenzoate, method A = 100) (i) 2.6, (ii) 1.3, and (iii) 3.9. The values obtained when the compounds and the arsenical were administered by the same route were (i) 68.5, (ii) 144, and (iii) 65.1. Effects other than surface activity alone were thus operative. Table VI also shows the trypanocidal titre *in vitro*, denaturant effect on protein, and approximate critical micelle concentration (estimated from surface tension/concentration curves in 1% serum-saline).

Only feeble surface activity was shown by the related compounds ethyl *p*-aminobenzoate, sulphanilic acid, *p*-aminophenol, benzoic, *p*-hydroxybenzoic, *p*-toluic, *p*-nitrobenzoic, phenylacetic and β-phenylpropionic acids, and by benzoylglycine and phenylglycine.

TABLE VI

PROPERTIES OF SURFACE-ACTIVE COMPOUNDS ANTAGONISTIC TO BUTARSEN

Where the effective interfering dose (EID) differs from the LD10, the latter is shown in brackets; critical micelle concentrations are estimated from surface tension/concentration curves in 1% serum/saline; N.D. = not determined.

Compound	Interference Activity/Mole (*p*-Aminobenzoic Acid = 100)	Effective Interfering Dose (M^{-1})	Depression at EID (Dynes/cm.)		Trypanocidal Titre ($\times 10^{-3}$)	Lowest Conc. for Protein Interaction (M^{-1})	Approximate Critical Micelle Concentration (M^{-1})
			1% Serum/Saline	0·85% Saline			
Palmitamidine	258	320	5·9	0	4,000	5,120	5,120
Octadecylamine	144	40	—	>5	2,000	640	640
Cetyltrimethylammonium bromide	68·5	100 (200)	17	18·4	64	5,120	5,120
Tauroglycocholic acid	65·1	80 (40)	22·9	25·2	1	>10,240	160
Trimethyloctadecylammonium chloride	48·3	160	10·3	3·4	256	10,240	640
Octadecamethylene diamine	34·8	320	8·1	—	16,000–64,000	640	—
Ricinoleic acid	*ca.* 20	20	>32·3	>35	4	>20,480	5,120
Lauric ,,	*ca.* 20	20	12	—	4	N.D.	—
Palmitic ,,	18·2	20	—	—	4	,,	—
Octadecylguanidine	2·6	46	>5·5	2·8	500	20,480	—
Trimethylnonylammonium iodide	0	160	>3·7	—	64	N.D.	—

The Effect of Aminobenzoate on the Action of Trypanocides of Varying Type

These results are shown in Table VII. The "inhibition time" in Tables VII and VIII is the difference between the average time of persistence of trypanosomes in undiminished or increasing numbers in the peripheral blood of a batch of four or five mice after intraperitoneal doses (0.5 ml./20 g.) of (a) the trypanocide (ED90), and (b) the interfering agent followed by the trypanocide. "Complete" inhibition means an infection course indistinguishable from that in untreated mice.

The results were much more variable with the metal-free and pentavalent arsenical trypanocides than with the trivalent arsenical drugs. The two first groups exert their action much more slowly (5 to 24 hr.) than the trivalent arsenicals in which visible trypanocidal action develops within 1 hr. of treatment.

p-Aminobenzoate interferes unequivocally only with atoxyl and two carboxyl-substituted arsenicals, butarsen and 3-acetamido-4-carboxyphenylarsenoxide. Significant antagonism by *p*-aminobenzoate is observed also with acriflavine, arsacetin (sodium *p*-acetamidophenylarsonate) and tryparsamide.

A wider range of examples of selective interference *in vivo* is shown in Table VIII, which

TABLE VII

EFFECT OF *p*-AMINOBENZOIC ACID ON THE ACTION OF TRYPANOCIDES OF VARYING TYPE

Drugs marked with an asterisk were as used by Yorke and Murgatroyd (1930); †, from Dr. F. Hawking; ‡ Indicates sodium 4-acetamido-3-hydroxyphenylarsonate.

Type	Compound	Dose (mg./kg.)	Inhibition Time (hr.)
Metal-free	Para rosaniline	16	0–19
	Acriflavine	10	19–37
	Suramin	2·7	0–20
	Pentamidine	0·65	0–20
As^{3+}	4-Carboxyphenylarsenoxide	15	13–18
	3-Acetamido-4-carboxyphenylarsenoxide	8·3	Complete
	Butarsen	0·75	"
	Arsenophenylglycine†	32·5	4–16
	Oxophenarsine	0·75	2
	Reduced tryparsamide*	4·0	2–14
	Dichlorophenarsine (halarsol)*	1·25	1
	Melarsen oxide	0·15	0·5
	Neoarsphenamine	6·25	1
As^{5+}	Atoxyl	150	45
	Arsacetin*	200	20–24
	Tryparsamide	750	19–22
	Orsanine‡	81·5	1–16
	Melarsen	30	3–11

TABLE VIII

SELECTIVE INTERFERENCE WITH TRYPANOCIDAL ACTION *IN VIVO*

Results marked with an asterisk refer to glutathione; † to cysteine.

Interfering Agent	Conc. (M^{-1})	Inhibition Time (hr.)			
		Maphar-side	Butarsen	Melarsen Oxide	Pent-amidine
Thiol	5	1*	1*	1·5*	0†
p-Aminoben-zoic acid	1	2	Complete	1	0–24
Surfen C	55	0	0	5–24	—
	110	—	—	5–24	—
	220	—	—	2–5·5	—
Melamine	4–12	0	0	Complete	24–48
Ammeline	12–24	0	0	1	0
Cyanuric acid	12–24	0	0	0	0
Tretamine	640	—	—	0	—
Surfen	47	1–6	1–5	4	—
	94	—	—	4	—
	188	—	—	2·5	—

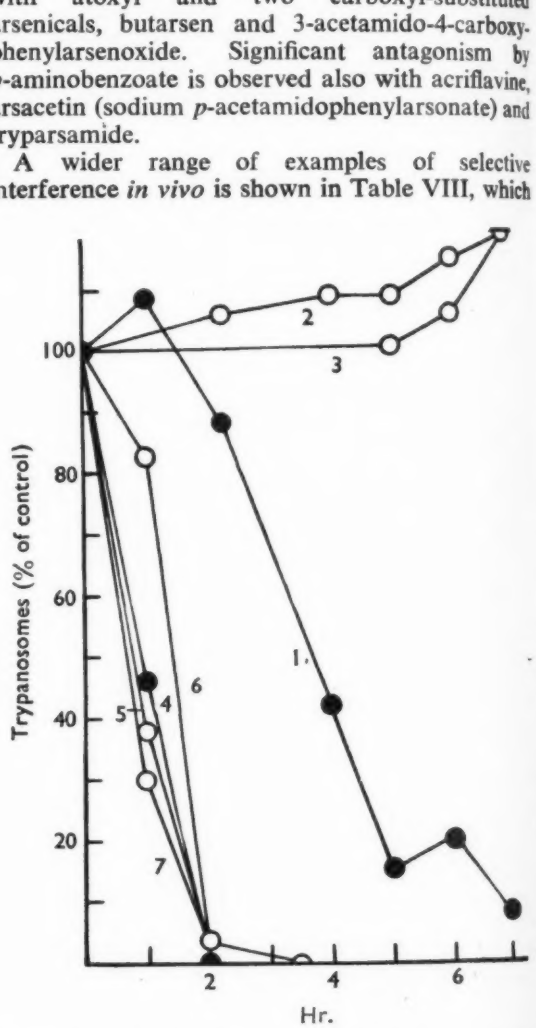

FIG. 2.—Selective interference with the trypanocidal action of melarsen oxide *in vitro*. Medium: 50/50 rabbit serum/0·85% NaCl-0·2% glucose. Temperature: 37°. Trypanosomes: about 1×10^6/ml. The ordinate represents the number of motile surviving parasites as a percentage of the control (medium only). Curves 1–3 from experiment 1. Curves 4–6 from experiment 2. Melarsen oxide 1: 102×10^8 present in all instances. 1 and 4, melarsen oxide alone; 2, also melamine 1: 6,080; 3, also cysteine 0.01 M; 5, also ammeline 1: 6,040; 6, also cyanuric acid 1: 5,930; 7, also *p*-aminobenzoic acid 1: 290.

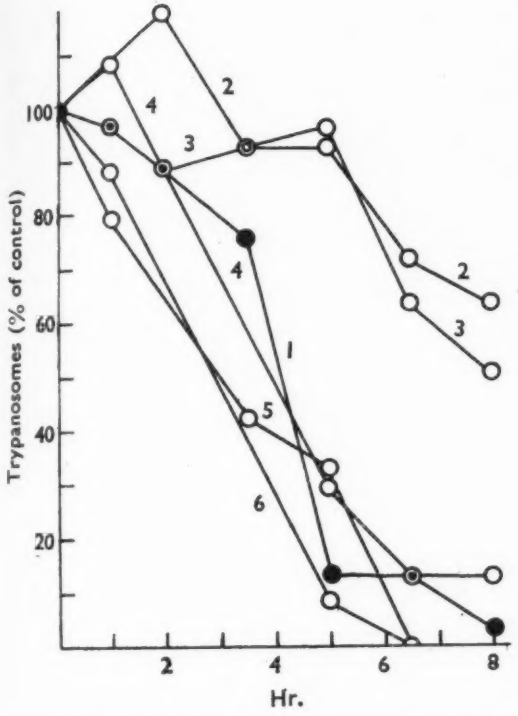

FIG. 3.—Selective interference with the trypanocidal action of pentamidine *in vitro*. Medium : 50/50 rabbit serum/0.85% NaCl-0.2% glucose. Temperature: 37°. Trypanosomes: approx. 1×10^6/ml. The ordinate represents the number of motile surviving parasites as a percentage of the control (medium only). Pentamidine 1: 1.6×10^6 was present in all instances. **1**, pentamidine alone; **2**, also melamine 1: 6,080; **3**, also cysteine 0.01 M; **4**, also ammeline 1: 6,040; **5**, also cyanuric acid 1: 5,930; **6**, also *p*-aminobenzoic acid 1: 290. The interference in **3** is probably due to salt formation.

shows that (a) thiol antagonism is common to all three types of aromatic arsenical but does not occur with pentamidine, (b) *p*-aminobenzoate antagonism is specific for the carboxylated arsenical butarsen, and (c) melamine antagonism is common only to the melaminyl arsenical, melarsen oxide, and to pentamidine.

Melamine antagonism shows structural specificity, because three closely-related substances, ammeline (cyanuric acid diamide), cyanuric acid, and tretamine, are inactive ; three compounds with amidine or guanidine groups (*p*-chlorophenylguanidine, proguanil and palmitamidine) are inactive against pentamidine.

Melamine (M/12) shows the same marked inhibition time (24 to 48 hr.) with decane diamidine, Synthalin B and stilbamidine, as with pentamidine. Melamine antagonism of melarsen oxide and pentamidine is also demonstrable *in vitro* (Figs. 2 and 3). The unexpected antagonism of pentamidine by cysteine *in vitro* is probably caused by salt formation, which could also explain the partial antagonism by *p*-aminobenzoate *in vivo*. The formation of crystalline salts with carboxylic acids is characteristic of the diamidine series (Walker, 1949), and a precipitate forms immediately on mixing dilute neutral solutions of pentamidine and sodium *p*-aminobenzoate.

DISCUSSION

Four likely mechanisms for chemotherapeutic interference may be distinguished: (a) chemical reaction between the drug and its antagonist with formation of an inactive product, (b) oxidation (or reduction) by the antagonist of a substance or redox system which is reduced (or oxidized) by the drug, (c) competition by the substrate for an enzyme inhibited by the drug, which is usually structurally related to the substrate concerned, and (d) with colloidal materials, formation of a biologically inert mixed micelle of drug and antagonist.

A well-known example of mechanism (a) is the formation of inactive thioarsenites from the reaction of excess thiols with trypanocidal arsenoxides (Voegtlin, Dyer and Leonard, 1923). Von Jancsó and von Jancsó (1936) invoked mechanism (b) in their experiments on the reversal of the trypanocidal activity of phenylarsenoxides and phenylstibinoxides by a large number of varied compounds sharing the property of exhibiting a redox potential more positive than -0.06 V (*p*H 7.0). Mechanism (c) is closely related to the competitive inhibitions observed with isolated enzyme systems, where there is a direct relation between the concentrations of inhibitor and substrate, which are often closely related in structure. Sulphanilamide/*p* - aminobenzoate antagonism in certain bacteria (Woods, 1940) is a classical example. The fourth type of mechanism, (d), has been proposed by Albert (1949) for drugs or antagonists with colloidal properties.

Mechanisms (b) and (c) most probably occur at the parasite cell/medium interface, where the primary interaction between drug and cell takes place. The primary fixation of a number of organic arsenicals is known to be reversible (Hawking, 1938), and Yorke and Murgatroyd (1930) showed earlier that " arsenic-resistance " in trypanosomes is not towards arsenic itself but to the substituted phenyl radical to which the arsenic is attached. The constitution of the phenyl radical is responsible for the primary fixation of the drug ; resistance does not develop to inorganic arsenic compounds such as sodium arsenite. This primary drug fixation concept has been elaborated

by Clark (1933), King and Strangeways (1942), and by Mudd (1945). Mudd (1945) stressed the common *p*-aminophenyl structural basis of a number of biologically active compounds such as sulphonamides, antipyretics, local anaesthetic esters of *p*-aminobenzoic acid and organometallic antiprotozoal agents; to these may be added the antitubercular drug *p*-aminosalicylic acid (Youmans, Raleigh, and Youmans, 1947) which is also trypanocidal (Pick, 1950), and *p*-aminobenzenephosphonic acid which is antibacterial (Bauer, 1941; Kanitkar and Bhide, 1947; Thayer, Magnuson and Gravatt, 1953). The diversity of active substances with the above general configuration implies the existence of a common and widely-distributed cellular reaction site of complementary structure.

Postulation of a primary reversible combination of a drug with a structurally specific site on or in the trypanosome, followed by a separate lethal stage, is the basis of the argument to be developed here for a common mechanism for selective interference phenomena and resistance behaviour in at least four main types of trypanocide.

p-*Aminobenzoic Acid/Butarsen Interference*

Neither an extracellular chemical reaction between the two substances, nor a possible functioning of *p*-aminobenzoic acid as an accessory respiration catalyst, is probable. Veldstra and Havinga (1947) have in fact shown polarographically that no oxidation or reduction of *p*-aminobenzoic acid occurs at voltages up to 0.6 V or 2.8 V respectively, and they consider it unlikely that *p*-aminobenzoic acid can function in a redox system. As *T. rhodesiense* has not yet been cultured satisfactorily on synthetic media, the unequivocal demonstration of *p*-aminobenzoic acid as an essential metabolite for the parasite is not possible; it had no effect in synthetic maintenance media developed by Williamson and Rollo (1952). Surface activity determinations indicate that the interference effect is unlikely to result from mixed micelle formation.

Hawking (1938) demonstrated a phase of primary adsorption conforming to the Langmuir adsorption isotherm in the trypanocidal action *in vitro* of acriflavine. This interpretation, as Ipsen (1941) emphasizes in connexion with haemolytic activity, may be due rather to an alternative expression of a normal probability distribution with respect to heterogeneous factors, such as resistance, among the parasites. A similar demonstration in terms of the drug competition theory of Gaddum (1943) has been attempted here for the antagonism of butarsen by *p*-aminobenzoic acid. The results (Table I, Fig. 1), though necessarily imprecise, suggest that a competitive adsorption of *p*-aminobenzoic acid may occur; the reversibility, on dilution with serum, of the immobilizing action of high concentrations of *p*-aminobenzoic acid on trypanosomes *in vitro* (Williamson and Lourie, 1946), also implies an initial loose adsorption phase.

Table II shows that, of the seven compounds which produce marked inhibition of the trypanocidal action of butarsen, *p*-aminobenzoic acid is the only one which causes complete and permanent inhibition at low concentrations. Moreover, the interference activity of *p*-aminobenzoic acid is completely abolished by alteration of the relative positions of the substituent amino and carboxyl groups from the *para* to the *ortho* or *meta* configuration. There are no gross differences between the three isomers in respect of physical properties, and chemically they share the amphoteric properties of the amino acids, except that in water a larger proportion of the *meta* acid exists as dipolar ions.

The three homologues show no regular increase or loss of interference activity with increasing length of the aliphatic side-chain (Table II), and none is as active as *p*-aminobenzoic acid. There is no correlation with pK_a (carboxyl); any shift in pK_a value (carboxyl or amino group) is progressive as the series is ascended, and all four compounds are 99% ionized as anions at blood *p*H. Schueler (1947) has indicated that, by virtue of the *n*-butyric acid side-chain which could assume an incomplete hexagonal structure on the basis of the normal carbon bond angle, butarsen may exist in forms where there is a much reduced distance between the arsenoso and carboxyl groups. This distance is likely to be of the order of separation of the amino and carboxyl groups in *p*-aminobenzoic acid (6.7 Å: Roblin and Bell, 1943), and this factor, combined with the similarity in acid strength of the two compounds, may be responsible for the specificity of the interference. Ionization of the amino group is negligible at blood *p*H in this series, and replacement of this group largely abolishes interference activity; it is therefore probable that hydrogen bonding, rather than ionic interaction, occurs at this locus. The importance of the spatial factor is emphasized by the lack of activity of *p*-aminocinnamic acid and the relatively high activity of both *p*-aminobenzoylglycine and benzoylglycine.

Complete removal of the carboxyl group, as in aniline, reduces activity by about 90%. The

activity of aniline is nevertheless marked; interference activity is completely abolished only by (i) replacement of the carboxyl by the sulphonic acid group, as in sulphanilic acid which has a much higher acid strength, (ii) replacement by a second ring structure as in sulphadiazine, (iii) substitution by a weakly acid group as in *p*-aminophenol (pK_a (OH)=10.30), or (iv) substitution by another amino group as in *p*-phenylene diamine, which destroys the heteropolar nature of the molecule and increases the basic strength (pK_a (amino), 6.08).

The activity of atoxyl (*p*-aminophenylarsonate) is of especial interest, since it indicates that this substance may be adsorbed to some extent on the trypanosome surface in the pentavalent form. The use of higher concentrations than those indicated is, of course, limited by the trypanocidal action of the compound, but the molecular configuration bears a close resemblance to *p*-aminobenzoic acid and adsorption at the same site is probable. In addition to the close structural resemblance the ionization at blood *p*H of the polar groups is similar; pK_a (amino, atoxyl)= 3.97, pK_a (carboxyl *p*-aminobenzoic acid)=4.81, pK_a (amino, atoxyl)=2.05, pK_a (amino, *p*-aminobenzoic acid)=2.51.

The bisquinolyl compound Surfen interferes actively not only with butarsen but also with oxophenarsine, and is almost as active as Surfen C in antagonizing melarsen oxide. Aqueous solutions of Surfen are markedly colloidal, and the antagonism probably results from mixed micelle formation.

The results above clearly indicate that the inhibitory action of *p*-aminobenzoic acid on the trypanocidal action of butarsen is highly specific and demands, as far as the experimental procedures described here can ascertain, the optimal assembly of the following factors: (i) a benzene nucleus, (ii) a negatively charged substituent, the pK_a of which is approximately 5.0, (iii) a weakly positive substituent, preferably an amino group, in the *para* position to (ii), the pK_a of which is equal to or less than 2.5, and (iv) the existence, actual or potential, of an inter-charge separation distance of approximately 7 Å.

The inter-charge separation distance, it may be noted, corresponds to twice the separation distance between two alternate side-chains in the polypeptide chain of a protein in the β-keratin configuration (Springall, 1954).

The trypanocidal action of butarsen is exerted very rapidly and depends on the achievement of a peak blood concentration almost immediately after intraperitoneal injection, which is followed within a few minutes by a precipitous fall (Murgatroyd, Russell, and Yorke, 1934). Therefore, with therapeutic interference where trypanocidal activity has been delayed for hours, this activity cannot be due to a trypanocidal concentration of the drug in the surrounding blood; it suggests that the drug is adsorbed at a point other than the vulnerable metabolic site where it exerts its ultimate lethal action, and to which it migrates only when the loosely-held antagonist is eluted as a result of the decreasing external bulk concentration. As Hurst (1943) points out, the lack of trypanocidal activity in phenyl-*pp'*-diarsenoxide suggests that the site of ultimate lethal action is not directly accessible to the external drug phase, since the availability of the arsenoso group —As:O is increased in this compound. In the case of *p*-aminobenzoic acid, where complete and permanent abolition of trypanocidal activity is effected, the antagonist-receptor affinity is high and at least equal to that of butarsen. On the basis of the results obtained here, it is not possible to establish directly whether the antagonism takes place at the trypanosome surface or intracellularly, though the results with compounds showing incomplete interference suggest the latter site.

Interpretation of *in vivo* experiments on interference must take into account the operation of "detoxicant" mechanisms. Since *p*-acetamidobenzoic acid is inactive, the acetylation process cannot affect the interference mechanism in the case of *p*-aminobenzoic acid, though it may well accelerate the loss of activity in *p*-amino-substituted compounds showing incomplete interference. In the case of *p*-aminomethylbenzoic acid, by analogy with maphenide, the basic group is probably rapidly oxidized *in vivo*, thus contributing to the low activity displayed.

Operation of the β-oxidation mechanism is also unlikely in the case of *p*-aminobenzoic acid and its homologues, since β-(*p*-aminophenyl)propionic acid, which should oxidize to the active *p*-aminobenzoic acid, is found in fact to have less than 2% of the activity of *p*-aminobenzoic acid. Reduction mechanisms do not seem to be important *in vivo*, since *p*-nitrobenzoic acid, which is reduced to *p*-aminobenzoic acid, has no interference activity. Hydrolysis of aromatic acid amides occurs *in vivo* and might be expected to confer higher activity on *p*-aminobenzamide, but the activity of this compound is much less than that of *p*-aminobenzoic acid. Apart from affecting rates of excretion, it would seem unlikely that *in vivo* detoxicant processes influence trypanocidal interference.

Surface Active Antagonists of Butarsen

As this interference is demonstrable only when the drug and antagonist are given by the same route, it seemed unlikely that facilitated preferential adsorption on the trypanosome of these compounds occurred by virtue of their surface-active polar-non-polar configuration. As Table VI shows, there is no correlation between interference activity and surface tension depression in saline or serum-saline solutions, trypanocidal titre, and the ability to flocculate serum protein. For the more soluble compounds, minima in the surface tension/concentration curves in serum-saline solutions were used as approximate estimates of critical micelle concentration. At the concentration used in the interference experiments, all the compounds formed visibly colloidal aqueous solutions. Micellar aggregation has been reported for compounds such as bile salts (Rains and Crawford, 1953), and the importance of critical micelle concentration in the detergent and biological action of compounds like cetyltrimethylammonium bromide and related substances is discussed in detail by Glassman (1948), Colichmann (1950), and Pankhurst (1953). Although there is no clear correlation between interference activity and critical micelle concentration (Table VI), it seems highly probable that the effective concentration of the injected butarsen is lowered by non-specific physical admixture with micelles of the antagonist.

Effect of p-*Aminobenzoic Acid on Trypanocides other than Butarsen*

Table VII shows that *p*-aminobenzoic acid interferes most effectively with carboxylated phenylarsenoxides and with atoxyl, as might be expected from the considerations above. Antagonism by *p*-aminobenzoic acid of the trypanocidal action of atoxyl has also been noted by Moshkovskiy and Stoyanova (1947). Atoxyl has been shown above to interfere with butarsen, and *p*-aminobenzoic acid is known to interfere with the antibacterial action of atoxyl (Hirsch, 1942). The latter result was confirmed by Peters (1943), who emphasized the importance of the structural factor in this effect; other pentavalent arsenical drugs such as acetarsol (3-acetamido-4-hydroxyphenylarsonic acid) and tryparsamide had no antibacterial activity and the bacteriostatic action of atoxyl, like that of sulphanilamide, was reversed by procaine (2-diethylaminoethyl *p*-aminobenzoate) and by methionine, but not by *o*- or *m*-aminobenzoic acid or by methyl 3-amino-4-hydroxybenzoate.

The pentavalent aromatic arsenicals circulate in the blood for 5 to 24 hr. before the reduced forms accumulate at trypanocidal levels. During this period, the arsonic acid group in all five drugs tested will be ionized like the carboxyl group of *p*-aminobenzoic acid (Table IV), but only atoxyl possesses the necessary intact primary *p*-amino group. An analogous competitive inhibition has been described by Seaman (1952) for the aliphatic pentavalent arsenical, arsonoacetate, on the succinic dehydrogenase of *Tetrahymena geleii*, S, where combination with "carboxyl affinity points" occurs without affecting thiol groups.

The selective interference experiments described here and summarized in Table VIII, together with the cross-resistance analyses reported earlier (Williamson and Rollo, 1959) and the data integrated by Schnitzer and Grunberg (1957), enable further inferences on trypanocidal drug resistance mechanisms to be made.

Selective permeability has been invoked frequently in the past to explain acquirement of resistance in trypanosomes, but there is no convincing evidence for this, and it is difficult to find a basis in terms of physical diffusion to the complex and highly selective patterns of resistance of which the trypanosome is capable. The cell phenomenon of pinocytosis (Fell, 1958), which seems to operate for the absorption of fat by *T. lewisi* and *T. equiperdum* (Wotton and Halsey, 1957), is an additional impediment to acceptance of the permeability thesis.

The trypanocidal drugs considered here are all aromatic compounds carrying substituents which are either ionized at blood *p*H or are capable of hydrogen bonding. There are relatively few kinds of chemical grouping in the cell with which trypanocides are likely to form a durable attachment, but these groups are capable of sufficient diversity of arrangement to account for the degree of structural specificity which is observed in drug action, resistance and interference behaviour. Assumption of a structurally specific and reversible primary fixation stage and a separate subsequent lethal stage accords with the facts so far assembled. The nature of the cellular "receptor" groups will be considered in a later report, but it is reasonable to assume that the trypanocides which are ionized at blood *p*H will combine with oppositely charged ionized groups in trypanosome cell material. This is the basis of the "isoelectric point shift" resistance theory of Schueler (1947), who postulated that such a shift "would be indicative of changes in the relative number of

positive or negative charges (for example, free amino or carboxyl groups) of the constituent proteins of the trypanosomes, a change which might very greatly affect the fixation of ionizable drug molecules." Such an alteration in receptor distribution could result from changes in the degree of folding of protein polypeptide chains or of nucleic acids (Goldacre and Lorch, 1950; Alexander, 1952; Kopac, 1947).

Aromatic arsenical drugs are generally conceded to be lethal by virtue of covalent binding of trivalent arsenic to essential cellular thiol groups. Little is known of the nature of any similar specific lethal mechanism for metal-free trypanocides, except that thiol combination is unlikely to be involved; there is, for example, no thiol antagonism of acriflavine (Strangeways, 1937; Schleyer and Schnitzer, 1948; Ercoli, Gosford, Carminati, Kley and Schwartz, 1951) or of stilbamidine or suramin (Ercoli *et al.*, 1951; Sen, Dutta and Ray, 1955). Thus reversal of trypanocidal action by thiols can be used to differentiate two stages in the action of three chemically distinct groups of aromatic arsenical. Table VIII shows that thiols antagonize the neutral oxophenarsine, the anionic butarsen, and the cationic melarsen oxide equally, but do not interfere with pentamidine. Oxophenarsine is known to be antagonized selectively by methyl *p*-hydroxybenzoate (Schleyer and Schnitzer, 1948), but not by *p*-aminobenzoic acid or melamine. Butarsen is antagonized selectively by *p*-aminobenzoic acid but not by methyl *p*-hydroxybenzoate (Schnitzer and Grunberg, 1957) nor by melamine. Melarsen oxide is antagonized by melamine but not *p*-aminobenzoic acid. These antagonisms resemble the malonate reversal of thiol poisons on succinic dehydrogenase (Potter and DuBois, 1943), and the pyruvate protection of carboxylase inactivation by organic arsenicals (Stoppani and Actis, 1952).

Thus each type of arsenical, neutral, anionic and cationic, appears to have a different structurally specific initial attachment site on the trypanosome cell. This corresponds to the observed resistance behaviour where an oxophenarsine-resistant strain is sensitive to butarsen and to melarsen oxide, a butarsen-resistant strain is resistant also to oxophenarsine but not to melarsen oxide, and a melarsen oxide-resistant strain is resistant to oxophenarsine but not to butarsen.

However, there are several anomalies not easily explicable on the assumption of either modified, and therefore resistant, primary receptors or of a changed relative proportion of anionic and cationic receptors. The cross-resistance between the cationic acriflavine and the neutral drugs such as oxophenarsine and tryparsamide is an obvious example. Despite the possibility of common factors in other contexts such as antimitotic activity, involving combination with nuclear material where reaction with thiols may be important (Bell, 1958), the trypanocidal action of acriflavine, unlike the arsenicals, is not reversed by thiols. A change in the relative proportion of anionic and cationic receptors in a strain made resistant to an ionic trypanocide would be expected to produce an increased sensitivity to ionized drugs with a charge of the opposite sign. In fact, a strain fast to the cationic stilbamidine is fast also to the anionic dye pontamine sky blue 5BX; Schumacher and Schnitzer (1956) have reported a butarsen-fast strain of *T. equiperdum* which, unlike the butarsen-fast *T. rhodesiense* used here, became cross-resistant to the cationic drugs Mel B and stilbamidine.

The suggestive but obscure interrelationships of para rosaniline, butarsen, *p*-aminobenzoic acid and the acriflavine-atoxyl group, which Schnitzer and Grunberg (1957) have noted, cannot be illuminated further by the present results; in addition, these results do not demonstrate cross-resistance of butarsen to para rosaniline or antagonism of para rosaniline by *p*-aminobenzoic acid in such a clear-cut fashion as the experiments of Schumacher and Schnitzer (1956) and Schnitzer and Schumacher (1956).

On the information presented here, the "two-stage" theory of trypanocidal action accords reasonably well with the observed facts. The specific melamine antagonism of the melaminyl arsenicals and antimonials and the diamidines reflects the cross-resistance between the groups and suggests that a common receptor group is modified during the development of resistance. It may be noted that melamine, like the diamidines (Walker, 1949), is capable of forming crystalline salts with carboxylic acids (Steele, Glover and Hodgson, 1952), and that the melamine interference is unlikely to be due to a redox effect because polarographic measurements show that pentamidine does not have a redox potential between $+0.2$ and -1.9 V against a saturated calomel electrode (H. Campbell, personal communication). At blood *p*H, both melamine (pK_a= 9.0, Dixon, Woodberry and Costa, 1947) and pentamidine (pK_a=11.40) are fully ionized, and the melaminyl substituent of melarsen (Table III) is about 70% ionized as the cation.

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DRUG RESISTANCE IN TRYPANOSOMES; EFFECTS OF METABOLIC INHIBITORS, *p*H AND OXIDATION-REDUCTION POTENTIAL ON NORMAL AND RESISTANT *TRYPANOSOMA RHODESIENSE*

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A wide variety of metabolic inhibitors tested *in vitro* for trypanocidal activity on normal and drug-resistant strains of *Trypanosoma rhodesiense* showed no relation between acquired drug resistance and changes in specific enzymatic function. Oxidation-reduction potential is an important factor in trypanocidal action but is not obviously related to the development of resistance. The dependence on *p*H of the trypanocidal action of ionizing drugs against both normal and resistant trypanosomes supports the postulate that the development of resistance involves physical changes in cell structures associated with the uptake of drug.

The development of acquired drug resistance in pathogenic trypanosomes is likely to involve at least two changes in the parasite, (i) a modification in the protein surface structure associated with drug uptake, and (ii) a change in metabolism whereby drug-sensitive enzyme reactions are modified, by-passed or eliminated; (i) and (ii) may obviously be related.

Attempts to elucidate mechanism (i) by cross-resistance analysis and selective interference experiments have been described (Williamson and Rollo, 1959; Williamson, 1959); the ionic basis of this behaviour has now been investigated by measuring the effect of *p*H on the trypanocidal action *in vitro* of representative anionic, cationic and feebly ionized drugs on normal and resistant parasites.

Little evidence exists for mechanism (ii) although changes in dehydrogenase activity of a melarsen-resistant *Trypanosoma rhodesiense* have been described (Williamson, 1953a). With the advent of a stilbamidine-fast strain (Fulton and Grant, 1955) which is resistant to a very wide variety of trypanocides (Williamson and Rollo, 1959) and would therefore be expected to show metabolic changes most obviously, a preliminary examination was made of its response to a number of metabolic inhibitors (Williamson, 1953b). Some differences, of a fairly low order, from the normal response were detected in the groups of phosphorase and dehydrogenase inhibitors; the differential action of some of these compounds has now been studied further by examination of the individual enzymatic processes likely to be involved.

As pathogenic African trypanosomes appear to rely on dehydrogenase rather than metalloporphyrin oxidase systems, and as some kinds of trypanocidal drug action are known to be affected by oxidation-reduction potential (von Jancsó and von Jancsó, 1936; Brownlee, Goss, Goodwin, Woodbine and Walls, 1950), this factor has been examined in detail electrometrically and by the use of redox indicators. Marked differences occurred in the reaction of normal and resistant strains, but although oxidation-reduction potential was apparently related to trypanocidal activity it was not obviously connected with the development of resistance.

METHODS

Trypanosome Strains.—The strains of *T. rhodesiense* and the tests of trypanocidal activity *in vivo* were as described (Williamson and Rollo, 1959).

Metabolic Inhibitor Experiments.—(i) Twenty-four different "specific" enzyme inhibitors were tested *in vitro* under standardized conditions against the normal and stilbamidine-resistant strains of *T. rhodesiense*. The "trypanocidal titre" of each compound was determined by the technique of Yorke

and Murgatroyd (1930), as described by Williamson and Rollo (1959), using a standard incubation period of 4 hr. at 37° (and, in some instances, also of 24 hr.). A mixture of equal parts by volume of fresh sterile horse serum and an aqueous solution containing 0.85% NaCl and 0.2% glucose was used as a supporting medium. The titre, for comparative purposes, has been expressed here as the reciprocal of the lowest molar concentration which lysed or immobilized at least 90% of parasites. Fourfold serial dilutions were made throughout and, where differences in reaction from the normal were detected in the resistant strain, experiments were repeated using serial dilutions with a factor of 2. A difference of one dilution-level can be considered significant within any one experiment, where the same inhibitor solution was titrated simultaneously against equivalent numbers of parasites of both strains in dilution series of four to six tubes. The short incubation period was chosen in order that inhibitor activity on trypanosome motility and viability could be roughly compared with that known to be exerted on other cellular or cell-free systems. The " resistance factor " is given by the ratio of the lowest trypanocidal concentrations for the resistant and normal strain respectively.

(ii) For experiments on phosphate metabolism, suspensions of normal and resistant trypanosome strains were prepared from infected blood obtained by cardiac puncture of heavily infected rats. The parasites were washed and suspended in a glucose-Ringer-phosphate saline containing 50% sterile inactivated horse serum, and the concentration was measured in a counting-chamber. The composition of the saline was as follows: 150 mM-NaCl, 11 mM-glucose, 0.2 mM-$MgCl_2$, 0.2 mM-K_2SO_4, 2.67 mM Sörensen phosphate buffer *p*H 7.4. Volumes of 3 ml. in capped sterile centrifuge tubes were incubated at 37° in a constant temperature water-bath. After centrifugation, 25% trichloroacetic acid was added to the parasite-free supernatant medium (1 vol. to 2 or 3 vol. of medium). After centrifuging again, 1.0 ml. samples of the protein-free supernatant were analysed for inorganic phosphate by the method of Gomori (1942); total phosphate was obtained by digestion according to Moraczewski and Kelsey (1948). Acid-soluble phosphate in trypanosome deposits was determined by disrupting the cells with 0.45 ml. of acetone followed by 24 hr. shaking at room temperature with 5 ml. of 5% trichloroacetic acid.

(iii) For respiration experiments, O_2 uptake in air was measured in conventional Warburg flasks at a shaking rate of 120/min., using a total volume of 3.0 ml. of parasite suspension and 0.15 ml. of 10% KOH in the centre well. At the end of the incubation period (30 min. at 37°) the contents of the flasks were transferred by Pasteur pipette to centrifuge tubes and, after centrifugation, the parasite-free supernatant fluid was analysed for phosphate. Parasite homogenates were prepared according to McIlwain (1948) by grinding with " Microid " alumina; all operations, from blood collection onwards, were carried out as far as possible at 0° to 5°. 2,4-Dinitrophenol and NaF solutions were made up in the medium for suspending parasites.

(iv) For experiments on citrate metabolism, washed erythrocyte-free suspensions of normal and stilbamidine-resistant strains of *T. rhodesiense* were prepared by differential centrifugation of heparinized infected blood taken by cardiac puncture from heavily infected rats. Homogenates were prepared by the method of McIlwain (1948). The suspending medium was a solution of 1% (v/v) sterile inactivated horse serum in a buffered glucose saline consisting of 116 mM-NaCl, 8.9 mM-glucose, 197 μM-$MgCl_2$, 230 μM-K_2SO_4 and 13.3 mM Sörensen phosphate buffer *p*H 7.4, the whole medium containing 333 μM-sodium citrate. Aerobic incubates (3 ml.) were set up for a period of 3 hr. in capped sterile tubes at 37° in a constant temperature water-bath, and parasite concentrations were determined with a counting chamber. Solutions of sodium fluoroacetate were prepared freshly in the supporting medium.

Analyses of the citrate content of the medium were made by the method of Pucher, Sherman and Vickery (1936) as modified by Buffa and Peters (1950), and the results are expressed as μg. of anhydrous citric acid. All reagents were A.R. grade, and the light petroleum (67–70° boiling fraction; May & Baker) used in the extractions was redistilled and washed with acid (M.A.R. concentrated H_2SO_4) several times, followed by washing with water, drying and redistillation.

After incubation, 1.0 ml. of 25% trichloroacetic acid was added to each tube, and the tubes were put in ice for 15 min. The precipitate was filtered off, washed twice with 5% trichloroacetic acid, and the filtrate was then boiled for 1 min. with 0.3 ml. of 50% H_2SO_4; subsequent analytical steps were as described by Buffa and Peters (1950).

Oxidation-Reduction Potential Experiments.—(i) The trypanocidal titres of fourteen reversibly reducible dyes were determined as described above for the metabolic inhibitors, except that the medium consisted of a mixture of equal volumes of sterile inactivated horse serum and Sörensen phosphate-buffered saline (*p*H 7.4); the buffer contained 0.2% glucose.

(ii) Electrometric determinations were made on erythrocyte-free trypanosome suspensions prepared by differential centrifugation of blood taken by cardiac puncture of heavily infected rats. The parasites were washed and finally suspended in a medium consisting of a mixture of equal volumes of M/15 Sörensen phosphate buffer *p*H 7.4 and an aqueous solution of 0.85% NaCl and 0.2% glucose; the whole contained 1% (v/v) sterile inactivated horse serum. Parasite concentration was checked with a counting chamber and the suspensions were incubated in $4\frac{1}{2} \times 1$ in. rimless tubes in a constant-temperature water-bath at 37°. Three tubes were set up simultaneously, each containing about 15 ml. of suspension at a parasite concentration of approximately 10^7/ml. and each fitted with a bung carrying a platinum electrode, an

agar-KCl bridge, gas inlet and outlet tubes, and a glass stirring rod. A source of O_2-free N_2 was connected through a water-bubbler to the gas inlet, and the free ends of the salt bridges, together with a saturated calomel electrode, dipped into a vessel containing saturated KCl solution. A Cambridge *p*H meter used as a millivoltmeter, with a wandering crocodile clip connection to the platinum electrodes, measured the potential in each incubation tube as required. At the end of the incubation period, the *p*H of the suspensions was checked with the glass electrode, and a drop was examined microscopically for parasite motility.

Effect of p*H on Trypanocidal Action* in Vitro.—The trypanocidal titre of the test compounds after 4 hr. incubation at 37° was measured as described above, but in media of controlled *p*H. The drugs were dissolved in an unbuffered medium consisting of a mixture of 1 part by volume of sterile inactivated horse serum and 9 parts by volume of glucose saline (0.85% NaCl and 0.2% glucose). Serial dilutions were made in a similar serum medium consisting of 1 part by volume of horse serum and 9 parts by volume of a 50/50 (v/v) mixture of glucose saline and M/15 Sörensen phosphate buffer. Before use, the *p*H of the resultant buffered serum-saline mixtures was checked with the glass electrode.

TABLE I

TRYPANOCIDAL ACTIVITY OF ENZYME INHIBITORS ON NORMAL AND STILBAMIDINE-FAST *T. RHODESIENSE IN VITRO* AT 37°

Incubation period, 4 hr., except for the numerals in parentheses. In these experiments, incubation was for 24 hr.

Site of Inhibition	Inhibitor	Trypanocidal Titre (M^{-1})	Resistance Factor
Metallo-oxidases	Cyanide	32	1
	Azide	128	1
	2,2′-Dipyridyl	512	1
	Hydroxylamine	320	1
Metal ion co-factors	Dithizone	5,120	1
	Oxine	786,000	1
	Fluoride	32	1
Dehydrogenases	Urethane	12	(2)
	Pentobarbitone	32	(0·25)
	Thiopentone	128	(1–0·25)
Thiol enzymes	Iodoacetate	32,800	1
	$HgCl_2$	2,050	1
	Arsenite	32,800	1
	Tetrathionate	128	1
Phosphorases	Alloxan	64	(4)
	1,2-Naphthaquinone-4-sulphonate	8,190	1
	Ninhydrin	2,050	2
	Pyrophosphate	32	1
	Phlorrhizin	32	1
	2,4-Dinitrophenol	128	2
	Methylene blue	131,000	4
Cholinesterase	Eserine	2,050	1
Amine oxidase	Ephedrine	512	1
Pyruvate utilization	Malonate	6	1
	Fluoroacetate	5	(0·5)
—	Pentamidine	237,000	16
	Melarsen oxide	30,000,000	64

RESULTS

Table I shows the activity *in vitro* of 24 different "specific" enzyme inhibitors against normal and stilbamidine-fast strains of *T. rhodesiense*. The inhibitors are grouped as far as possible according to their specific reactivities. Some differential action against the resistant strain is apparent in the dehydrogenase and phosphorase inhibitor groups, and also with fluoroacetate. The relatively high trypanocidal activity of oxine suggested that this might be reversed by cobaltous ions in the same way as its antibacterial action (Albert, Rubbo, Goldacre and Balfour, 1947); Table II shows that this specific reversal of trypanocidal action does occur.

TABLE II

REVERSAL BY Co^{2+} OF THE TRYPANOCIDAL ACTION OF 8-HYDROXYQUINOLINE (OXINE) ON *T. RHODESIENSE IN VITRO* AT 37°

Incubation period, 4 hr.

Metal Ion	Trypanosome Numbers (as % Control)	
	Metal Ion (M/393,000)	Metal Ion (M/393,000) + Oxine (M/786,000)
Ca^{2+}	147	5·6
Mg^{2+}	128	<1
Mn^{2+}	120	<1
Cd^{2+}	0	0
Fe^{2+}	92	<1
Co^{2+}	114	117
Zn^{2+}	139	<1
Ni^{2+}	100	0
Cu^{2+}	128	0
Medium only	100	
Oxine M/786,000	<1	<1

Experiments on phosphate metabolism are recorded in Tables III, IV and V. All suspensions (concentration: 10^8 to 10^9 trypanosomes/3 ml.), with negligible variants, produced an increase in the inorganic phosphate of the medium, which originally contained about 6 μmole of orthophosphate. Neither the addition of NaF nor an increase in the Mg^{2+} content of the medium to 0.02 M appreciably inhibited this shedding of

TABLE III

LOSS OF INORGANIC PHOSPHORUS FROM STATIC TRYPANOSOME SUSPENSIONS AT 37°: LACK OF EFFECT OF NaF AND 2,4-DINITROPHENOL

+ Indicates an increase and − a decrease in phosphorus content of the medium compared with the control (parasite-free medium or suspension at the start of incubation period).

Inhibitor Added	Change in Medium Inorganic P Content (μmole/10^9 Trypanosomes/hr.)		
	Mean	No. of Expts.	Range
None	+0·55	11	(−0·39 to +1·6)
NaF (3 to 10 mM)	+0·86	6	(−0·5 to +1·5)
2,4-Dinitrophenol (0·75 to 2·5 mM)	+0·87	4	(0 to +2·0)

TABLE IV

CHANGES IN TRICHLOROACETIC ACID-SOLUBLE PHOSPHORUS OF TRYPANOSOMES IN STATIC SUSPENSIONS AT 37° IN RELATION TO CHANGES IN PHOSPHORUS CONTENT OF MEDIUM

No. of Parasites/ 3 ml. ($\times 10^{-6}$)	Change in P Content of Medium (μmole/10^9/hr.)			Trichloroacetic Acid-soluble P in Trypanosomes (μmole/10^9)					
				At 0 hr.			Change after 1 hr.		
	Inorganic	Organic	Total	Inorganic	Organic	Total	Inorganic	Organic	Total
900	+0·84	+3·3	+4·14	2·1	2·7	4·8	−0·65	−2·7	−3·35
984	+1·16	+1·05	+2·21	1·34	2·07	3·4	−0·99	−0·52	−1·51

phosphate by the trypanosomes; 2,4-dinitrophenol was similarly without effect on the normal or the resistant strain (Table III).

Two experiments, in which the trichloroacetic acid-soluble (organic) phosphate of the trypanosome deposits was determined before and after incubation, indicated that the increase in the phosphate content of the medium is caused largely, if not entirely, by irreversible breakdown and loss of the labile intracellular organic phosphate in the trypanosomes (Table IV). The conditions of preparation of the suspension, and of abnormal cultural environment and population density, are most probably responsible for this loss.

Static suspensions of trypanosomes were used in the experiments detailed in Tables III and IV. Table V shows the results of experiments with

TABLE V

LOSS OF INORGANIC PHOSPHORUS FROM SHAKEN TRYPANOSOME SUSPENSIONS AT 37°; LACK OF EFFECT OF 2,4-DINITROPHENOL

N indicates normal strain; and R, stilbamidine-fast strain. O_2 uptakes are the means of five experiments, except that marked with an asterisk, which is the mean of three experiments.

2,4-Dinitrophenol Conc. (μM)	Strain	No. of Expts.	Increase in Medium Inorganic P (μmole/10^9 Trypanosomes/hr.)	O_2 Uptake (μmole/ 10^9 Trypanosomes/hr.)
0	N	10	+1·55 (−3·49 to +4·97)	50·0
	R	6	+1·74 (−1·94 ,, +5·68)	49·4
47	N	5	+0·26 (−3·49 to +3·04)	51·1
	R	6	+2·26 (0 ,, +3·68)	47·8
188	N	6	+1·94 (0 to +6·66)	40·5*
	R	7	+1·81 (−1·94 ,, +4·06)	49·7
750	N	6	+2·46 (0 to +3·42)	53·3
	R	7	+2·13 (−3·87 ,, +4·06)	49·8
3,000	N	5	+1·16 (0 to +3·81)	50·8
	R	5	+0·39 (−1·94 ,, +2·0)	40·6

shaken suspensions. In these experiments with the Warburg respirometer in which O_2 uptake was also measured, the shedding of inorganic phosphate into the medium by the shaken suspensions (concentration: 10^8 to 10^9 trypanosomes/3 ml.) was about twice as great as that observed with static suspensions. The presence of 2,4-dinitrophenol (47 to 3,000 μM) had no noticeable effect on this loss, and there was no detectable difference between normal and resistant strains. In several experiments the changes in absolute amounts of phosphate were small and at the lower limits of precision of the analytical method used; this accounts for the variability of the results and the apparent phosphate uptake in some instances.

The effect of 2,4-dinitrophenol on O_2 uptake is shown in Table VI. The suspension concentration in these ten experiments ranged from 170 to

TABLE VI

EFFECT OF 2,4-DINITROPHENOL ON THE O_2 UPTAKE IN AIR OF SUSPENSIONS OF NORMAL AND STILBAMIDINE-RESISTANT STRAINS OF *T. RHODESIENSE* AT 37°

N indicates a normal strain, and R a stilbamidine-resistant strain.

2,4-Dinitrophenol Conc. (μM)	Strain	qO_2/10^9 Parasites in Expt. No.					Mean Change (%) from Control qO_2
		1	2	3	4	5	
	N	1,555	1,540	1,162	746	590	
	R	1,648	1,295	907	916	762	
47	N	1,860	1,386	1,172	700	604	+1·4
	R	1,678	1,114	893	913	751	−3·1
188	N			1,332	771	615	+7·5
	R	1,832	1,243	891	894	704	−0·8
750	N	1,860	1,395	1,424	669	628	+5·8
	R	1,730	1,264	916	932	740	+0·5
3,000	N	1,850	1,332	1,176	795	542	+1·1
	R	1,265	1,074	846	777	628	−15·9
Parasite concentration/ 3 ml. ($\times 10^{-6}$)	N	170	221	427	646	902	
	R	272	510	629	629	765	

902×10^6 trypanosomes/3 ml., and an inverse relationship was observed between respiratory rate and parasite concentration. In a series of 26 experiments in which the parasite concentration varied from 64×10^6/3 ml. to $1{,}248 \times 10^6$/3 ml. the corresponding respiratory rate range (qO_2/10^9 trypanosomes) was 2,196 to 311, which is similar to that observed by von Brand and Tobie (1948). The respiratory rate throughout is based on the uptake observed during the first 30 min. of the experimental incubation period.

There was some stimulation of O_2 uptake by 2,4-dinitrophenol at the lower drug concentrations and at the lowest parasite concentrations, especially on the normal strain. The O_2 uptake of the resistant strain tended rather to be inhibited; this inhibition was most marked, by comparison with the normal strain, at a concentration of 3 mM 2,4-dinitrophenol. The difference in response (17%) is significant; using Student's t test, the significance level is 0.02 to 0.05.

Two experiments with homogenates were made in the Warburg respirometer. Each flask contained 3.0 ml. of a dilution of homogenate in the standard medium equivalent to 350×10^6 and 560×10^6 trypanosomes respectively in the two series. In both, microscopic examination of the homogenates showed almost complete cell disruption. Over a 30 min. incubation period, the O_2 uptake of these homogenates was negligible. The inorganic phosphate content of the medium was determined after protein precipitation by tipping in 1.0 ml. of 25% (v/v) trichloroacetic acid from the side-arm of the reaction flask at varying intervals. No phosphate uptake was demonstrable at incubation periods of 15 min and 30 min. in the presence or absence of 3 mM 2,4-dinitrophenol.

Table VII, which gives the results of experiments on citrate metabolism, shows clearly that there is no aerobic utilization of citrate, and no accumulation of citrate in the presence of fluoroacetate, either in whole suspensions or in cell-free preparations of normal or stilbamidine-resistant strains of *T. rhodesiense*.

Table VIII shows the trypanocidal titre of fourteen reversibly-reducible substances including twelve "redox indicators." The values quoted for the oxidation-reduction potential of the half-reduced substances (E'_o) are for *p*H 7.0 and temperature 30°. In the case of janus green, the final reduction stage is well defined, corresponding to $E'_o = -0.255$ V, but an intermediate blue-red change corresponding to $E'_o = -0.035$ V is quoted by von Jancsó and von Jancsó (1936). Electrometric titration with hydrosulphite under N_2 has shown a red-blue change in the neighbourhood of 0 V followed by a sharp end-point at the -0.25 V level; there are thus no grounds for supposing, as Page and Robinson (1943) have done, that the B.D.H. product used by them (and in the present work) differs essentially from the dye used by von Jancsó and von Jancsó (1936). The E'_o value used here is -0.035 V.

From these results, trypanocidal activity is obviously not a simple function of oxidation-reduction potential, but, with the exception of Bindschedler's green, the eight most highly active compounds have E'_o values in the region $+0.15$ V to -0.15 V.

TABLE VII

EFFECT OF FLUOROACETATE ON CITRATE METABOLISM OF NORMAL AND STILBAMIDINE-RESISTANT *T. RHODESIENSE*

Incubate	Citric Acid after 3 hr. at 37° (μg.)			
	Normal Strain		Resistant Strain	
	Free Parasites	Homogenate	Free Parasites	Homogenate
Medium	192	192	192	192
,, +parasites	201	183	183	192
,, + +M/120" fluoroacetate	183	178	183	183
Parasites/3 ml. ($\times 10^{-6}$)	32	21	32	90

TABLE VIII

TRYPANOCIDAL ACTIVITY *IN VITRO* AT 37° OF REVERSIBLY-REDUCIBLE TEST COMPOUNDS AGAINST NORMAL AND STILBAMIDINE-RESISTANT *T. RHODESIENSE* (*p*H: 7·4)

An asterisk indicates drugs obtained from British Drug Houses Ltd., redox indicator. †, British Drug Houses Ltd., standard stain. ‡, G. T. Gurr Ltd. §, Braid and Co. Ltd.

Test Substance	E'_o (V)	No. of Expts.	Trypanocidal Titre (M^{-1}) at		Resistance Factor	
			4 hr.	24 hr.	4 hr.	24 hr.
Ferricyanide	+0·43	2	<1,000	2,000–8,000	1	1
Alloxan	+0·406	2	32–64	128	1	4
*Bindschedler's green (Zn double salt) [C.I. No. 819 (1st Edn.); 49405 (2nd Edn.)] ..	+0·224	2	64,000	64,000–256,000	1	1
*o-Cresol-2,6-dichlorophenol-indophenol ..	+0·181	1	8,000–32,000	32,000	1	
*Toluylene blue (C.I. No. 820; 49410) ..	+0·115	1	8,000	32,000	1	1
‡Thionine (C.I. No. 920; 52000)	+0·063	1	32,000	512,000	4	4
†Brilliant cresyl blue (C.I. No. 877; 51010)	+0·047	3	32,000	512,000	4	1
§Toluidine blue (C.I. No. 925; 52040) ..	+0·011	2	32,000	128,000	1	1
†Methylene blue (C.I. No. 922; 52015) ..	+0·011	4	128,000	2,000,000	4	4
†Janus green (C.I. No. 133; 11045) ..	−0·035	3	512,000	33,000,000	1	1
†Nile blue (C.I. No. 913; 51180) ..	−0·122	4	32,000	512,000	1	
†Indigo carmine (C.I. No. 1180; 73015) ..	−0·125	4	8,000	32,000	4–16	16
*Phenosafranine (C.I. No. 840; 50200) ..	−0·252	4	2,000	128,000	4	1
‡Neutral red (C.I. No. 825; 50040) ..	−0·325	2	2,000	32,000	1	1

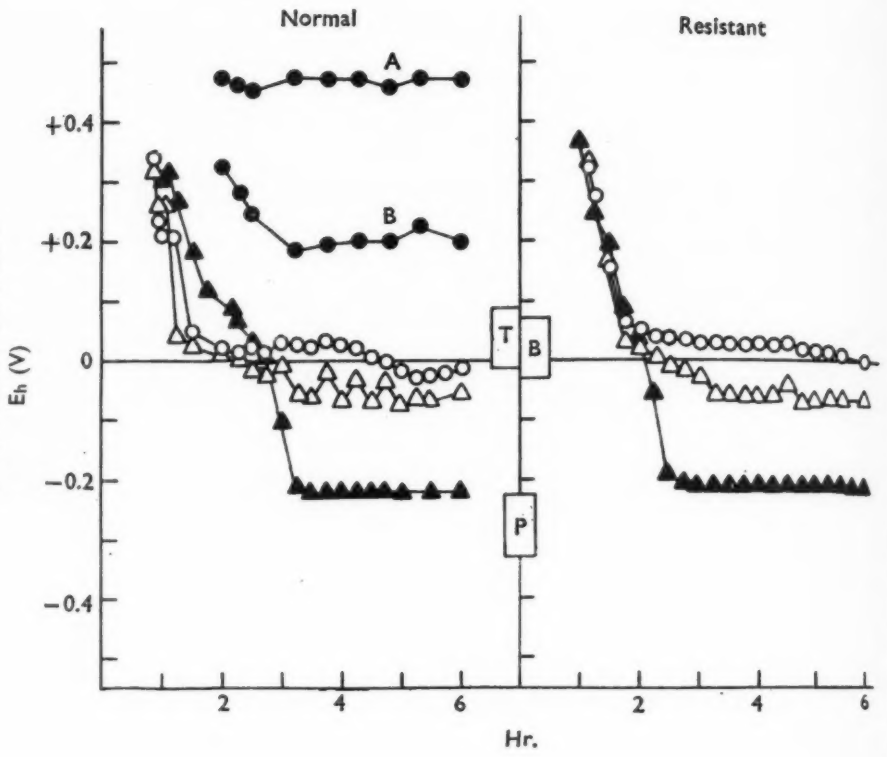

FIG. 1.—Effect of redox dyes on the oxidation-reduction potential of suspensions of normal and stilbamidine-resistant *T. rhodesiense* at 37° and *p*H 7·4 in O_2-free N_2. ● **A**, medium only; ● **B**, suspension only; **O**, thionine (**T**, M/8,500); **Δ**, brilliant cresyl blue (**B**, M/133,000); **▲**, phenosafranine (**P**, M/8,300). Blocks on central ordinate show 2 to 98% reduction zone for **T**, **B**, and **P**.

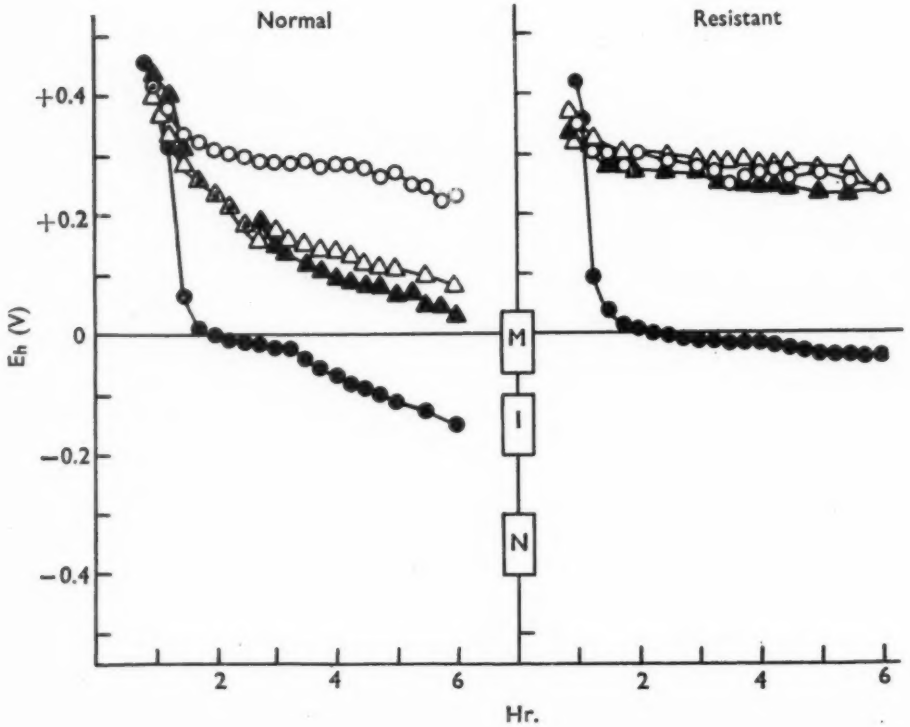

FIG. 2.—Effect of redox dyes on the oxidation-reduction potential of suspensions of normal and stilbamidine-resistant *T. rhodesiense* at 37° and *p*H 7.4 in O_2-free N_2. **O**, neutral red (**N**, M/8,300); **Δ**, indigo carmine (**I**, M/8,300); **▲**, indigo carmine (M/33,000); ●, methylene blue (**M**, M/8,300). Blocks on central ordinate show 2 to 98% reduction zone for **N**, **I**, and **M**.

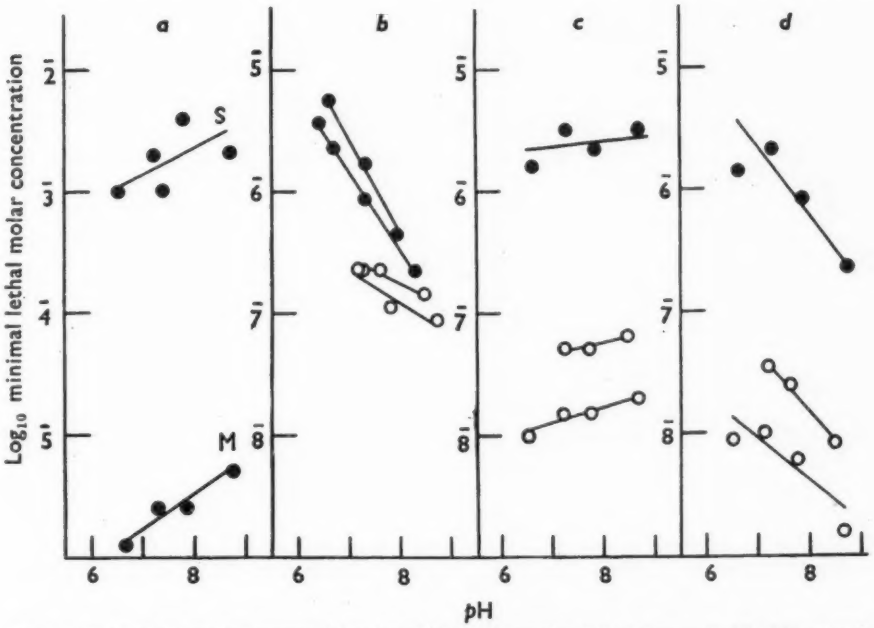

FIG. 3.—Effect of *p*H on the trypanocidal action of (*a*) oxophenarsine (M) and suramin (S), (*b*) pentamidine, (*c*) melarsen oxide, and (*d*) melarsen oxide as cation, against *T. rhodesiense in vitro* at 37°. Incubation time, 4 hr. O, Normal strain; ●, strain resistant to drug under test.

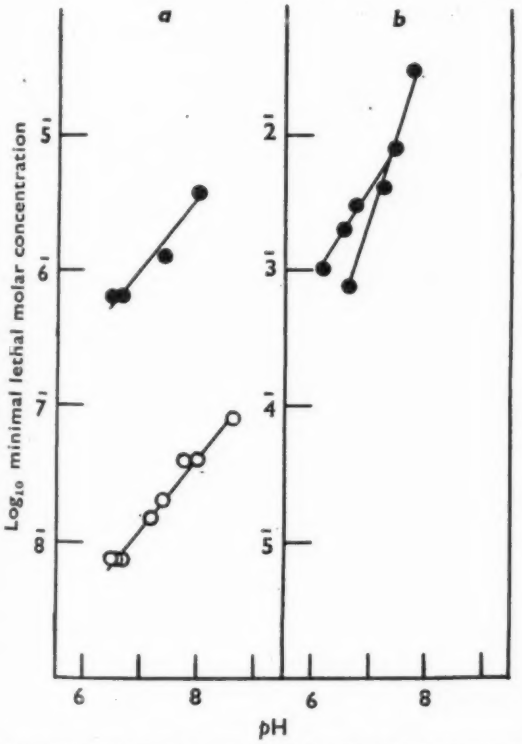

FIG. 4.—Effect of *p*H on the trypanocidal action of (a) butarsen and (*b*) indigo carmine, against *T. rhodesiense in vitro* at 37°. Incubation time, 4 hr. O, Normal strain; ●, strain resistant to drug under test.

In the graphs of results of electrometric measurements in trypanosome suspensions (Figs. 1 and 2), the ordinate represents the measured oxidation-reduction potential (E_h, referred to the normal H_2 electrode) at 37° and *p*H 7.4, and the abscissa represents time of incubation in hr., zero being the time of withdrawal of infected blood for the preparation of the suspension. Blocks on a central ordinate indicate the 2 to 98% reduction range of potential of the indicators used at *p*H 7.4 and 37° ($E'_o \pm 0.051$ V); the indicators were those to which the stilbamidine-resistant strain showed some resistance *in vitro*.

A few initial experiments with aerobic suspensions showed variable and erratic potentials, but anaerobically the potentials were stable and reproducible. The medium alone showed a steady level of about +0.47 V, and a trypanosome suspension without added dyes showed a fall of about 0.25 V from this level in the first 2 to 3 hr. of incubation followed by a slower decline to a level of about +0.2 V after 6 hr. This probably reflects the initial rapid O_2 uptake of the parasites followed by progressive decline and the increasing liberation of reducing substances by dying cells (Fig. 1).

The dependence on *p*H of the trypanocidal action *in vitro* of pentamidine, melarsen oxide, butarsen and indigo carmine is shown in Figs. 3 and 4.

The trypanocidal action *in vitro* of oxophenarsine and suramin was less dependent on *p*H (Fig. 3*a*) as might be expected from the lack of actively ionizing substituents in oxophenarsine and the relative inactivity *in vitro* of suramin, the mode of action of which is unlikely to be similar to that of the other drugs in the group tested.

Discussion

Few of the metabolic inhibitors listed in Table I can be considered specific for one given enzyme reaction, but several share a common chemical property, such as the ability to combine with or inactivate thiol groups, or to form chelate complexes with metal ions, which can reasonably be taken to underlie any observed inhibitory or lethal effect. The details of metabolism in *T. rhodesiense* have been established as yet only fragmentarily, and a full interpretation of inhibitor action can be made only by methods involving chemical determination of intermediate substances formed in the interrupted metabolic chain. Nevertheless, specific inhibitors showing trypanocidal activity in high dilution may be valuable indicators of the presence of a given enzyme reaction; conversely, any striking lack of lethal effect in low dilution may indicate the absence of a particular enzyme, though here the interpretation can be complicated by the cell-permeability factor.

The insensitivity of the respiration of pathogenic African trypanosomes to cyanide is well known (von Brand and Johnson, 1947; von Brand and Tobie, 1948) and is taken to indicate the absence of metalloporphyrin oxidases; this is reflected in the results here. Cyanide definitely stimulates motility, possibly by stimulation of anaerobic glycolysis or, as von Brand and Tobie (1948) have indicated, by keto-fixation (for example, cyanhydrin formation with pyruvic acid, the main end-product of glycolysis). The somewhat greater inhibition with azide may be connected with its ability also to inhibit phosphorases.

2,2′-Dipyridyl chelates strongly at blood *p*H specifically with Fe^{2+} ions, and markedly inhibits the anaerobic glycolysis of *T. lewisi* (Ryley, 1951). It has a less marked but appreciable action on *T. rhodesiense*; hydroxylamine has the same order of activity.

Several divalent metal ions in trace amounts are specific co-factors for enzyme reactions, for example, Mg^{2+} for enolase, Fe^{2+} and Cu^{2+} for the oxidation of ascorbic acid and glutathione respectively. At *p*H 7.3 dithizone combines specifically with Zn^{2+}, Co^{2+}, Pb^{2+}, Cu^{2+} and Cd^{2+} ions, and oxine with Mn^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+}, Fe^{2+} and Fe^{3+} ions (Albert and Gledhill, 1947). Fluoride inactivates the enzyme enolase by combination with Mg^{2+} ions. Both dithizone and oxine have a marked trypanocidal action, especially the latter, the trypanocidal properties of which were observed by Liese (1928). Since the antibacterial action of oxine is due to the formation of toxic complexes with Fe and Cu^{2+} ions (Albert *et al.*, 1947; Rubbo, Albert and Gibson, 1950; Albert, Gibson and Rubbo, 1953) and is reversible by Co^{2+} ions, the possibility that a similar mechanism might operate in its trypanocidal activity was investigated. Of nine divalent metal ions tested (see Table II) only Co^{2+} reversed the trypanocidal action of oxine (at the concentration used, Cd^{2+} ions were toxic). The protective action of Co^{2+} on the metal-catalysed oxidation of a group vital to metabolism, as indicated by Rubbo *et al.* (1950), may thus operate in *T. rhodesiense*; this is not improbable in view of the major importance of glycolysis to the parasites requiring the participation of thiols such as glutathione which is easily oxidized in the presence of metal ions (Ryley, 1953). The value of M/786,000 for oxine activity was unchanged by further incubation up to 24 hr. and seems to indicate that the trypanosomes are more sensitive than Gram-positive bacteria to oxine, if M/100,000 is regarded as the characteristic antibacterial concentration. This may reflect the relatively greater dependence of pathogenic trypanosomes on intact thiol groups if the action of oxine involves metal-catalysed thiol oxidations.

Central nervous system depressant drugs are relatively specific dehydrogenase inhibitors, and *T. rhodesiense* is known to possess dehydrogenase systems. Urethane and pentobarbitone (Nembutal) have relatively little effect, though the sulphur analogue of the latter, thiopentone, shows appreciable activity. However, it is possible that the effect is developed over the longer incubation period (24 hr.), when the resistant strain shows a resistance to urethane and a sensitivity to pentobarbitone and thiopentone, compared with the normal strain. This is not unexpected in view of the differences in dehydrogenase substrate activation already demonstrated between normal and melarsen-resistant strains of *T. rhodesiense* (Williamson, 1953a).

Inhibitors of thiol enzymes act by alkylation, mercaptide formation or oxidation, and their high degree of trypanocidal activity reflects the well-known importance of functional thiol groups in *T. rhodesiense* metabolism; tetrathionate, which inhibits by oxidation, is not, however, markedly

active. Some overlap with other inhibitor types occurs in this group, as iodoacetate is a specific inhibitor of triosephosphate dehydrogenase, and probably alloxan and naphthaquinonesulphonate, and possibly phlorrhizin, which are phosphorase inhibitors, owe this action to thiol inactivation.

The term "phosphorase" is the generic term coined by Dixon (1951) to include enzymes "which put on or remove or transfer phosphoric acid groups." The participation of phosphate in *T. rhodesiense* metabolism is likely by analogy with other pathogenic trypanosomes in which a phosphorylative glycolytic process has been shown, and by implication from the results in Tables III, IV and V.

1,2-Naphthaquinone-4-sulphonate and ninhydrin show marked relative trypanocidal activity and are presumed to act on thiol enzymes of the phosphokinase type. The presence of this type of enzyme in *T. evansi* and its inhibition by arsenoxide has been shown by Marshall (1948). Pyrophosphate and phlorrhizin are relatively inactive, but alloxan and 2,4-dinitrophenol show appreciable activity.

The main interest in this group is the differential action of four inhibitors on the resistant strain. The latter shows resistance to methylene blue, 2,4-dinitrophenol, ninhydrin and alloxan. 2,4-Dinitrophenol and methylene blue interfere with oxidative phosphorylation, and this property is shared by several other dyestuffs and some amidine and guanidine compounds, including trypanocidal derivatives. Alteration of drug-sensitive systems such as these might be expected to occur in the resistant trypanosome strain, but the results in Tables III, IV, V and VI show that phosphate uptake is not demonstrable under the experimental conditions here, and that there is no differential action of 2,4-dinitrophenol on the resistant strain.

Eserine is a specific inhibitor of cholinesterase and, since acetylcholine has been demonstrated in *T. rhodesiense* (Bülbring, Lourie and Pardoe, 1949), some eserine inhibition might be expected. The activity here is not marked (2,050 compared with approximately 10^6 on cholinesterase).

Ephedrine is a specific inhibitor of amine oxidase, which is inhibited by diamidines. Protein metabolism in *T. rhodesiense* has not been extensively investigated and is probably small *in vitro*, although amino-acid utilization occurs (Williamson and Rollo, 1952). Ephedrine inhibition here is marked but not outstanding.

Malonate and fluoroacetate are inhibitors of component reactions of the Krebs cycle involved in the utilization of pyruvate. Since pyruvate is the main end product of *T. rhodesiense* glycolysis, utilization is probably negligible, though it may occur to a small degree (Ryley, 1956) and is likely in *T. evansi* (Marshall, 1948). Malonate, a specific inhibitor of succinic dehydrogenase, has no significant inhibitory activity. Fluoroacetate, which inhibits the aconitase reaction and promotes citrate accumulation, is likewise relatively inactive, though some activity is developed over the longer incubation period. A differential action is, however, shown against the resistant strain which develops some sensitivity to the inhibitor. Guanidine compounds, including the trypanocidal Synthalin B, can cause citrate accumulation in animals and tissue preparations (Buffa, Peters and Wakelin, 1951), and it has been found that the melarsen-resistant strain develops marked citrate dehydrogenase activity not shown by the normal strain (Williamson, 1953a). Nevertheless, Table VII shows that there is no significant citrate utilization or accumulation in *T. rhodesiense* suspensions and no differential effect of fluoroacetate on the stilbamidine-fast strain. Ryley (1956) in experiments on *T. rhodesiense* showed that citrate was produced by this trypanosome in serum-free media.

A number of trypanocides such as phenylarsenoxides, acriflavine, stilbamidine and several dyestuffs have been shown to act as mitotic poisons. For this reason, and also because one of the more recently-developed radiomimetic compounds, tretamine (triethylene melamine; used in the treatment of leukaemia and Hodgkin's disease), is based on the melamine structure which appears to be involved in the action of the diamidine and melamine arsenical trypanocides, a number of radiomimetic compounds were tested for trypanocidal action, with the aim of relating further specific metabolic inhibitions to resistance development.

Tretamine, as noted earlier, has no trypanocidal activity. Two other active compounds of this sort, busulphan [Myleran; 1:4-di(methanesulphonoxy)butane], used in the treatment of myeloid leukaemia, and 8-azaguanine (carcinostatic for certain mouse tumours, and assumed to be a guanine antagonist), were tested, but showed no trypanocidal activity. Colchicine, the antimitotic action of which, like arsenicals, appears to be confined to suppression of spindle-formation, is similarly inactive as a trypanocide. All tests were made in living mice, as these compounds are primarily active against dividing cells.

So far as drug resistance is concerned, the above results with metabolic inhibitors would seem to

indicate that, in the stilbamidine-fast strain, the development of a high degree of resistance to a wide variety of trypanocides is not generally associated with comparably gross changes in intermediary metabolism. This conclusion accords with the inferences of Williamson and Rollo (1959) and of Williamson (1959) that resistance involves a steric modification of specific structures associated with the preliminary reversible adsorption stage of trypanocidal drug action.

A further aspect of drug-resistance capable of experimental investigation emerged from the work with enzyme inhibitors. Many of the latter act by irreversibly oxidizing or reducing susceptible cell components operating in a narrow range of oxidation-reduction potential. Oxidation-reduction changes in resistant cells were therefore investigated in two ways: (a) by measurement of the overall trypanocidal activity of twelve different oxidation-reduction indicators covering a potential range of $+0.43$ V to -0.34 V, and (b) by direct electrometric measurement of the oxidation-reduction potentials of O_2-free suspensions of parasites in the presence and absence of selected indicators.

Table VIII shows the results of investigation (a). The twelve indicator dyes used, with the exception of indophenol and indigo carmine, have superficially similar structures. They are either derivatives of diamino-substituted phenazines, oxazines or thiazines, or of indamine (Bindschedler's green and toluylene blue), but these similarities are not sufficiently close to isolate oxidation-reduction potential as a determinant activity factor. Using a more varied range of dyes, Page and Robinson (1943) showed a complete absence of correlation between oxidation-reduction potential and antibacterial activity against *Staphylococcus aureus*. Inspection of the antibacterial titres recorded by these workers for ten dyes used in the present study reveals, however, that the trypanocidal is much greater than the antibacterial activity, and that the most active antibacterial dyes have E'_o values between $+0.05$ V and -0.12 V, which is within the range recorded above for maximal trypanocidal activity (Table VIII). Although, in this restricted series of dyes, lethality *in vitro* for *T. rhodesiense* and *S. aureus* appears to be related to oxidation-reduction potential, a number of examples may be cited to show that high trypanocidal activity is not necessarily associated with the possession of an E'_o value in the region of 0 V: (i) the aminoacridine, acriflavine, which has a trypanocidal titre (M^{-1}) of 6.6×10^7 (Yorke and Murgatroyd, 1930), has a strongly negative E'_o value of about -0.4 V (Breyer, Buchanan and Duewell, 1944); (ii) a number of trypanocidal phenylarsonic acids examined polarographically show strongly negative half-wave potentials of -1.26 V to -1.47 V against the normal calomel electrode (Breyer, 1939); and (iii) trypanocidal aromatic diamidines, in which the aromatic nuclei are joined by a saturated chain and are substituted by groups which in themselves do not possess an oxidation-reduction potential, are not reduced between $+0.2$ V and -1.9 V against the saturated calomel electrode at the dropping mercury electrode (H. Campbell, personal communication).

The stilbamidine-resistant strain is resistant *in vitro* to the phenothiazine dyes—thionine and methylene blue, to the phenoxazine dye—brilliant cresyl blue, to the phenazine dye—phenosafranine, and to the sulphonated indigo dye—indigo carmine; resistance to alloxan has already been noted above. The cross-resistance to these dyes is not related to their oxidation-reduction potential, and in fact recapitulates in another context the early observations of Ehrlich, Gonder, Werbitzki and others (summarized by Morgenroth, 1914) on the cross-resistance between certain aromatic arsenicals and dyes derived from the pyronine, acridine, selenopyronine, phenoxazine, phenothiazine and phenoselenazine series, in trypanosome strains made resistant to one or other of the two groups. The tricyclic anthracene-type nucleus, with one or two heteroatoms in the meso positions, characterizes dyes of the type listed above to which resistance is shown, but is present only in an incomplete form in the indophenol and indamine dyes, to which no resistance is shown here. Nor is the strain resistant to two other structurally divergent dyes, janus green, in which an aminophenazonium salt similar to phenosafranine is connected by an azo-linkage to dimethylaniline, and nile blue, which is a naphthophenoxazine. No resistance is shown to the phenothiazine dye toluidine blue or to the phenazine dye neutral red, both of which are closely related to methylene blue and to brilliant cresyl blue. The relatively high degree of cross-resistance to indigo carmine is of particular interest, since this is the only acid-substituted dye in the group tested and the possession of substituents able to ionize as anions at blood *p*H has generally been correlated with activity against resistant strains of the type used here. Some elucidation of this result is afforded by the electrometric experiments discussed below.

Some other biological reactions of a number of dyes used here may be considered. Several are well-known respiratory accelerants for a wide variety of cells and tissues, and, in fact, von Jancsó and von Jancsó (1936) suggested that the redox dyes which interfered with trypanocidal action did so by acting as accessory electron carriers for oxidations poisoned by the drug. Dickens (1935) found that phenosafranine was a highly potent inhibitor of the Pasteur effect in normal and tumour tissues, and Dickens (1936a, 1936b, 1939) showed similar activity in a number of related dyes. The ability of these dyes to raise aerobic glycolysis towards anaerobic levels was not correlated with respiratory acceleration, but there was a tendency for the more electropositive dyes to be inactive or to increase the Pasteur effect. Of particular interest is the inclusion of the trypanocides acriflavine, Synthalin B and undecane diamidine, and of "Styryl 430" in the group of highly active inhibitors. More recently, and more specifically, this inhibitory capacity has been correlated with depression of aerobic phosphorylation in cell-free tissue preparations (Judah and Williams-Ashman, 1951) and in rat tissues *in vivo* and *in vitro* (Dianzani and Scuro, 1956). A possibly related effect, where degradation of cozymase is inhibited, is restricted among the dyes tested to basic, quaternary dyes of the phenosafranine type (McIlwain, 1950; McIlwain and Grinyer, 1950). A comparison of these various dye reactions is presented in Table IX. The experimental materials are diverse: Dickens worked with rat kidney and brain slices, and rat and mouse tumour tissue, McIlwain (1950) with mammalian brain and spinal cord tissue extracts and guinea-pig brain slices, and Judah and Williams-Ashman (1951) with cell-free rabbit and rat kidney and liver particulate fractions. As the latter workers have emphasized, results on cell-free and intact cell preparations cannot be strictly compared since, in addition to the complex of molecular properties determining dye access to whole cells, species and tissue specificity factors are involved. No obvious parallelism with trypanocidal activity or cross-resistance behaviour can be discerned, but the association of Pasteur effect inhibition and trypanocidal activity is clearly marked with acriflavine, "Styryl 430," the trypanocidal aliphatic diamidines and diguanidines, and with dyes such as janus green. Trypanosomes of the *T. brucei* group have a high rate of aerobic glycolysis; further increase, with increased acid production, might be toxic, as Dickens (1939) has suggested. It may be significant, also, that the structural requirements postulated for inhibitory activity in the Pasteur reaction (Dickens, 1936b) apply to most of the few compounds (phenanthridine, cinnoline and quinaldine quaternary salts) found to be active against the arsenic-refractory *T. congolense*, where the aerobic glycolysis rate, though marked, is not so high as in the *T. brucei* group (von Brand, 1951).

TABLE IX

SOME BIOLOGICAL REACTIONS OF DYES AND TRYPANOCIDES

Columns marked with an asterisk indicate values obtained from Dickens (1936a, 1936b, and 1939); †, McIlwain (1950), McIlwain and Grinyer (1950); ‡ Judah and Williams-Ashman (1951). § Refers to guinea-pig brain cortex slices (McIlwain and Grinyer, 1950).

Test Compound	Respiratory Acceleration*	Release of Aerobic Glycolysis*	Cozymase Degradation†	Depression of Aerobic Phosphorylation‡
Ferricyanide	−	−		
Bindschedler's green	−	−		
Toluylene blue		−		±
Thionine	++	−		+++
Brilliant cresyl blue	++	−	±	+++
Methylene blue	+		−	+++
Janus green	−	(+§)	+++	+++
Nile blue	−			
Phenosafranine	−	+++	+++	+++
Neutral red	−	−(+§)	+	±
Acriflavine	−	+++		
Undecane diamidine	−	+++	−	+++
"Styryl 430"		+++	−	

No aerobic reduction of twelve of the fourteen redox indicators (all those tested except alloxan and methylene blue) was observed at 37° with trypanosome preparations under the following conditions: (i) M/2,500 dyes in a mixture of equal amounts of horse serum and glucose-saline with heat-killed normal, living normal and stilbamidine-resistant *T. rhodesiense* at a concentration of about 10^7/ml. incubated up to 20 hr.; (ii) M/8,000 dyes in phosphate-buffered glucose-saline mixture (*p*H 7.4) containing water-lysed normal and stilbamidine-resistant *T. rhodesiense* (approximately 10^7/ml.) incubated up to 96 hr.

Anaerobic incubation at 37° in evacuated Thunberg tubes of (i) M/4,500 dyes in phosphate-buffered glucose-saline (*p*H 7.4) containing saponin-lysed normal and stilbamidine-resistant *T. rhodesiense* (1.4×10^6/ml.) showed no reduction up to 17.5 hr., and of (ii) dye concentrations equivalent to the minimum lethal concentration *in vitro* (at a parasite concentration of approximately 1×10^6/ml.), in phosphate-buffered glucose-saline (*p*H 7.4) containing living normal and stilbamidine-resistant *T. rhodesiense* (4.2×10^6/ml.) incubated up to 18 hr., showed some reduction of *o*-cresol-2,6-dichlorophenol-indophenol, brilliant cresyl blue and toluidine blue. In several instances, the minimum lethal concentrations *in*

vitro were below visible coloration levels, and electrometric measurements were undertaken to avoid this and other defects of the colorimetric method.

Both normal and resistant trypanosome suspensions took up potentials with thionine (M/8,500) and brilliant cresyl blue (M/33,000) characteristic of the 2 to 98% reduction range of these dyes (Fig. 4). With phenosafranine (M/8,300), both strains took up a steady potential (−0.210 to −0.225 V) characteristic of minimal dye reduction, and may represent the lower limit of reducing capacity of the suspensions under these conditions, since neutral red (M/8,300) with a more negative E'_o value is not reduced by either strain (Fig. 2). This lower limit of reducing capacity falls within the redox potential range of flavins and flavoproteins (Dixon, 1951) and may reflect the dependence of pathogenic African trypanosomes on this type of oxidase rather than on metalloporphyrin systems, as noted earlier.

Methylene blue (M/8,300) showed a differential action: with the normal strain the 2 to 98% reduction zone was passed through rapidly, and the suspension potential continued to become more negative, reaching a level of −0.165 V after 6.5 hr.; with the resistant strain, the suspension maintained a steady potential well within the 2 to 98% reduction zone (Fig. 2). As the dye concentration was near-toxic for the normal strain, the rapid fall in potential with this strain probably represented increased dye uptake followed by cell injury, death and release of reducing substances; with the resistant strain dye access was limited.

A differential action is more marked with the acid dye indigo carmine (indigo disulphonate) (Fig. 2). This dye was not reduced by either strain, but at concentrations of M/8,300 and M/33,000 a fall in potential of 0.3 to 0.4 V occurred with the normal but not with the resistant strain. The difference is not so marked at the less toxic concentration of M/133,000. Again dye access was probably involved; addition of saponin to the resistant strain suspension containing M/33,000 dye produced a rapid fall in potential of about 0.3 V, suggesting release of previously inaccessible reducing systems capable of reacting with the dye. Indigo carmine, unlike the other dyes tested here, is an acid ionized at *p*H 7.4 and it is probable that resistance to indigo carmine involves changes in the ionization of cationic receptors associated with dye uptake. Experiments on the effect of *p*H on trypanocidal action *in vitro* have shown that indigo carmine uptake by a resistant strain was *p*H dependent (Fig. 4).

Although oxidation-reduction potential was apparently related to trypanocidal activity, it was not in itself obviously related to the development of resistance, and the marked differences revealed with some indicators by the electrometric method can most satisfactorily be explained by alterations in dye transport across the cell wall. The electrometric method seems a particularly sensitive way of detecting such transport if the substances investigated are capable of reacting with indifferent electrodes.

The importance of ionization as a factor in the action of selectively toxic agents has been stressed by Albert (1951). Antibacterial diamidines and acridines are two drug series with trypanocidal members which have been investigated in this way. The dependence on *p*H of the antibacterial action of the former group has been noted by Bernheim (1943) and by Elson (1945); Fig. 3b shows that the trypanocidal action of pentamidine is similarly determined by *p*H. Since the pK_a of pentamidine is 11.4, the drug is 100% ionized over the *p*H range tested, and the observed increase in activity with increasing *p*H is most probably due to competition with H^+ for an anionic receptor group ionizing over this range, as has been suggested by Albert, Rubbo, Goldacre, Davey and Stone (1945) for the similar behaviour of antibacterial acridines. The reaction to pentamidine of the resistant strain is also *p*H-dependent over the same range as the normal strain. This parallelism implies that the same anionic receptor for pentamidine is functioning in both the normal and resistant strains, and that the reduced drug uptake by the resistant strains reflects a decrease in the relative proportion of these receptors. A change of this sort might produce a shift in the isoelectric point of the parasite protein, as envisaged by Schueler (1947), and it is hoped to investigate this point further by electrophoretic methods and by potentiometric titration of separated protein material.

Melarsen oxide (Fig. 3c) shows no marked dependence on *p*H, but recalculation of the minimum lethal concentrations of drug in terms of the cationic form present (the pK_a of the basic group in melarsen is 7.64) shows that the most active form of the drug is the cation, and that, as in pentamidine, these cations compete with H^+ for an anionic receptor (Fig. 3d). In view of the cross-resistance behaviour described earlier, and of the similarity of the *p*H-activity curves of pentamidine and of the cation of melarsen oxide, this anionic receptor may well be the same for both drug types, possibly the hydroxyl group of tyrosine or of nucleic acid purines and pyrimidines, as

suggested by Albert (1951) for antibacterial acidines.

The *p*H-activity curves of the anionic trypanocide butarsen (Fig. 4a) reveal a similar mechanism involving a cationic receptor ionizing in the *p*H 6 to 9 range for both the normal and resistant strains. Within this range, the butarsen molecule is present as an anion to the extent of 95 to 99.9%, and the observed increased activity with lowered *p*H is therefore less likely to be due to an increased proportion of the more freely cell-permeable non-ionized molecules, as Eagle (1945) suggests, than to a change in the extent of ionization of a cationic receptor. Two such possible receptors are the α-amino group of cystine and the imidazolyl group of histidine; the latter group is " generally responsible for most of the buffering power of proteins in the physiological *p*H range " and is the most important binding site for zinc and other ions in the case of serum albumin (Edsall, Felsenfeld, Goodman, and Gurd, 1954).

The trypanocidal action of indigo carmine is also *p*H-dependent (Fig. 4b). The available results refer only to a strain resistant to the dye, but as indigo carmine is fully ionized over the *p*H range tested, the variation in activity may reasonably be ascribed to the varying ionization of a cationic receptor.

These results lend further support to the thesis that acquired drug resistance in trypanosomes is a process which involves physical changes in specific cellular structures associated with preliminary drug uptake rather than alterations in drug-sensitive metabolic function.

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A COMPARISON BETWEEN THE EFFECTS OF EDROPHONIUM AND CHOLINE IN THE SKELETAL MUSCLES OF THE CAT

BY

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The effects of edrophonium and choline have been compared with those of the depolarizing substances acetylcholine, decamethonium, and suxamethonium, in both innervated and chronically denervated tibialis anterior muscles of cats under chloralose anaesthesia. Both edrophonium and choline were more potent antagonists to paralysis by tubocurarine than could be accounted for by their ability to stimulate the motor end-plates directly. It appeared likely that direct depolarization of the end-plate played no part in the anti-curare action of edrophonium and only some part in the anti-curare action of choline. A paralysis produced by the neuromuscular blocking agent, benzoquinonium, was more readily antagonized by a tetanus or by acetylcholine, suxamethonium, and decamethonium than a similar paralysis produced by tubocurarine. The tetraethyl ammonium ion was also slightly more effective against a paralysis by benzoquinonium. On the other hand, edrophonium was about 300 times and choline about five times less potent as an antagonist to benzoquinonium than to tubocurarine. Furthermore, the previous administration of benzoquinonium abolished the antagonistic action to tubocurarine of normally effective doses of edrophonium and reduced that of choline. These results were similar to those previously obtained with neostigmine, physostigmine and ethyl pyrophosphate and suggested that there was some similarity in the mechanism of action of all of these substances. Benzoquinonium, therefore, showed promise as a useful pharmacological tool for distinguishing compounds with this particular type of action. These anti-curare compounds did not appear to act by cholinesterase inhibition, nor by an increase in the sensitivity of the motor end-plates. In common with other workers, we suggest that there is a pre-synaptic mechanism of action.

The mode of action of the anti-curare substance edrophonium has not been fully established, but several mechanisms have been suggested, namely inhibition of cholinesterase (Hobbiger, 1952; Smith, Cohen, Pelikan, and Unna, 1952; Nastuk and Alexander, 1954; Katz and Thesleff, 1957), a direct acetylcholine-like action at the motor end-plates (Wescoe and Riker, 1951; Riker, 1953), and finally a direct action on the nerve endings (Riker, Roberts, Standaert, and Fujimori, 1957; Riker, Werner, Roberts, and Kuperman, 1959). According to Riker *et al.* (1957, 1959), edrophonium reacts with membrane receptors in the nerve endings causing the motor nerve to fire repetitively and thus increasing the transmitter action. A somewhat similar pre-synaptic mechanism of action had previously been proposed by Hutter (1952a) to account for part of the action of choline at the neuromuscular junction.

It was therefore decided to re-examine and compare the properties of edrophonium and choline in the hope that further light might be shed upon their mechanism of action.

METHODS

Cats, anaesthetized with chloralose (80 mg./kg. body weight) injected into the subcutaneous vein of the forelimb, were used throughout the experiments. For the experiments on innervated muscles, shielded silver electrodes were placed on the sciatic nerve of one hind-limb and the nerve was ligated centrally to the electrodes. The limb was set up in a horizontal position on a Brown-Schuster myograph stand, and the tendon of the tibialis anterior muscle was attached to a flat steel spring myograph. Twitches and tetani, which were recorded on smoked paper, were excited by rectangular pulses of 0.2 msec. duration and of twice the strength required to evoke a maximal twitch. For experiments on denervated muscle, the sciatic nerve was divided under sodium pento-

barbitone anaesthesia and degeneration was allowed to proceed for from 17 to 19 days.

Drugs were injected intravenously or close-arterially. For close-arterial injections the preparation described by Brown (1938) was used; all such injections were 0.2 ml. in volume. The drugs used were edrophonium chloride, choline chloride, acetylcholine chloride, tetraethylammonium chloride, suxamethonium chloride, and decamethonium iodide. The solutions were made in 0.9% saline. The doses quoted in the text refer to the quantity of the drug calculated as base. At the beginning of all experiments, atropine sulphate (1 mg./kg.) was administered intravenously.

Results

Stimulant Action

In the unstimulated innervated muscle, close-arterial injection of edrophonium (15 to 100 μg.), choline (0.3 to 1 mg.), decamethonium (0.3 to 1 μg.), and suxamethonium (0.5 to 1.5 μg.) caused, after a short latent period, marked fasciculations of the muscle. Larger amounts of choline (2 to 3 mg.), decamethonium (2 to 5 μg.), and suxamethonium (3 to 5 μg.) caused a quick contraction followed by an immediate relaxation. An increase of the same order in the dose of edrophonium (0.15 to 0.2 mg.) caused a quick contraction of the muscle, but this was followed by only partial relaxation after which powerful fasciculations occurred. With still larger doses (0.3 mg. and above) edrophonium caused a quick contraction which now was followed by a complete relaxation. With acetylcholine (1 μg. or more) a quick contraction followed by immediate relaxation was the only effect seen. Fig. 1 illustrates some of these results. Table I shows a comparison of the doses of all five substances necessary to produce a quick contraction of the muscle approximately equivalent to 50% of the maximal twitch tension.

TABLE I

RELATIVE POTENCIES WITH REFERENCE TO DECAMETHONIUM

The estimates are based on: (a) the doses required to produce an immediate contraction equivalent to about half the maximal twitch tension; (b) the doses required to produce contractures equivalent to about half the maximum; and (c) the smallest doses necessary to produce a distinct decurarization.

	Decamethonium	Acetylcholine	Suxamethonium	Choline	Edrophonium
(a) Innervated muscles ..	1	0·8–1	1·6	900–1,200	28–40
(b) Chronically denervated muscles ..	1	0·03–0·07	1·7	250–300	15–24
(c) Antagonism to tubocurarine	1	150–200	1·5–2	140–180	1·25–1·8

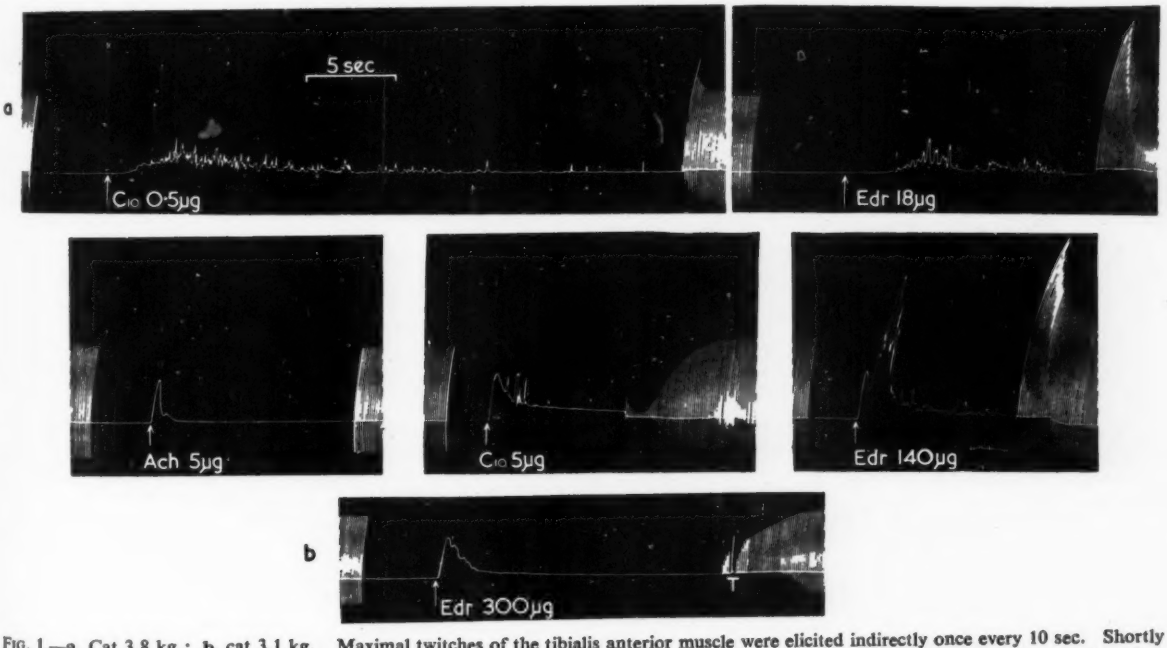

FIG. 1.—a, Cat 3.8 kg.; b, cat 3.1 kg. Maximal twitches of the tibialis anterior muscle were elicited indirectly once every 10 sec. Shortly before each injection, electrical stimulation was stopped and the kymograph speed increased. At C_{10}, decamethonium, at **Edr**, edrophonium, and at **Ach**, acetylcholine were injected close-arterially. At **T**, the sciatic nerve was stimulated at a frequency of 50/sec. for 15 sec.

In the chronically denervated muscle, choline and edrophonium, like decamethonium, suxamethonium and acetylcholine, caused a contracture on close-arterial injection. This effect of edrophonium was demonstrated by Randall (1950). A comparison of the potencies of all five substances is illustrated in Fig. 2. Table Ib shows the combined results of five experiments in which the doses required to produce contractures equivalent to approximately 50% of the maximum were determined.

It is well known that the sensitivity of skeletal muscles to many quaternary ammonium compounds, particularly to acetylcholine, is increased after chronic denervation. In the present experiments, the denervated tibialis anterior muscle became more sensitive to all four stable compounds but to different degrees. The increase in sensitivity was equal for decamethonium and suxamethonium but much greater for edrophonium and choline.

Effects on Maximal Twitch

Small doses of edrophonium cause potentiation of the maximal twitch, while large doses cause neuromuscular block (Randall, 1950). These results were confirmed in the present experiments. There was a striking difference, however, between the effects of edrophonium on the one hand and those of decamethonium, suxamethonium, and choline on the other. Edrophonium caused potentiation of the maximal twitch in doses as low as 2 to 3 μg. administered close-arterially, such doses being five to 10 times smaller than those necessary to cause fasciculations of the resting muscle. Furthermore, potentiation of the twitch tension, without any secondary depression, occurred over a wide range of doses (from 2 to 200 μg.), and only very large doses caused neuromuscular block (Fig. 1). Choline caused potentiation of the maximal twitch in doses approximately half the size of those required to cause fasciculations. The doses of decamethonium and suxamethonium necessary to produce potentiation of the maximal twitch were of the same order as those which caused fasciculations in the resting muscles. In contrast to edrophonium, the range of doses over which choline, decamethonium, and suxamethonium produced only potentiation was narrow, the onset of neuromuscular block occurring with doses only three to four times greater than the smallest necessary to cause potentiation.

Although all four substances caused neuromuscular block, its characteristics were not the same. During partial paralysis produced by edrophonium, tetanic tension was not sustained, and after the tetanus the paralysis was temporarily deepened (Fig. 1b). On the other hand, as Hutter (1952a) showed, paralysis produced by choline resembled that produced by decamethonium and suxamethonium; during the block, tetanic tension was well maintained, and after the tetanus transmission was neither restored nor further depressed.

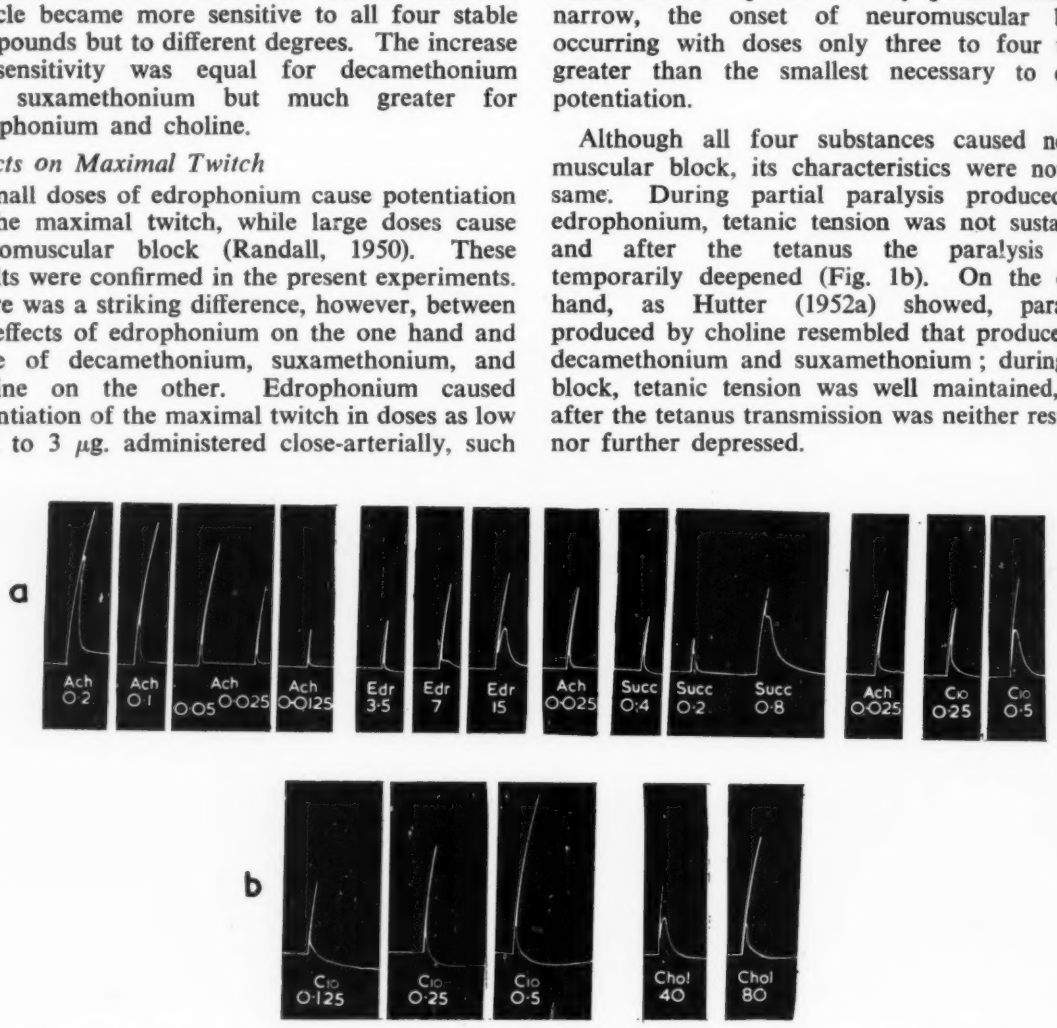

FIG. 2.—a, Cat 3.6 kg.; b, cat 2.8 kg. Chronically denervated tibialis anterior muscle (18 days). At **Ach**, acetylcholine; at **Edr**, edrophonium; at **Succ**, suxamethonium; at C_{10}, decamethonium; at **Chol**, choline. All injections were made close-arterially. The numerals denote the doses of the drugs in μg.

Anti-curare Action

Sufficient tubocurarine was administered intravenously to produce an 80 to 90% paralysis of the indirectly excited twitches and, at the peak of the blockade, choline, edrophonium, acetylcholine, suxamethonium, or decamethonium was administered close-arterially. Table Ic gives the ratios of the smallest doses capable of causing a distinct decurarization. There was little difference in the minimal effective anti-curare doses of edrophonium, suxamethonium, and decamethonium (Table I; Figs. 3 and 5). This was in marked contrast to their relative abilities to stimulate the resting innervated muscle, in which edrophonium was about 35 times less potent than decamethonium and suxamethonium.

The same was true for choline which, if compared with decamethonium and suxamethonium, possessed about 1/150 to 1/200 of their potency as anti-curare agents but was only about 1/1,000 as active in its ability to stimulate the resting muscle (Table Ia and c).

The results obtained with acetylcholine confirmed those of Hutter (1952a), who showed that large doses were required to antagonize the action of tubocurarine, and that the substance was, in fact, no more effective than a chemically equivalent amount of choline (Fig. 5).

Interaction with Benzoquinonium

Benzoquinonium is a potent neuromuscular blocking drug, very similar in most of its actions to tubocurarine (Hoppe, 1950, 1951; Bowman, 1958). There is, however, one important difference; in the cat, the blockade produced by benzoquinonium is not antagonized by the usual effective anti-curare doses of edrophonium (Randall, 1951). After the administration of benzoquinonium, the ability of normally effective doses of edrophonium to antagonize tubocurarine is abolished (Bowman, 1958). In the present experiments it was found that very large doses of edrophonium (0.5 to 1 mg. close-arterially) did antagonize the blockade produced by benzoquinonium although the antagonism was always preceded by a slight increase in the depth of paralysis (Fig. 3).

Choline to some extent resembled edrophonium in its interactions with benzoquinonium. The substance was more effective in antagonizing paralysis produced by tubocurarine than that due to benzoquinonium. Furthermore, the antagonism by choline of paralysis due to tubocurarine was reduced by the previous administration of benzoquinonium (Fig. 4).

There were, however, quantitative differences in the actions of edrophonium and choline. Paralysis produced by benzoquinonium was only antagonized by edrophonium in doses 300 times greater than those necessary to produce a similar degree of antagonism to tubocurarine (Fig. 3), and a single intravenous dose of benzoquinonium could completely prevent, for periods up to 2 hr., the anti-curare action of a previously effective dose of edrophonium (Bowman, 1958). Choline, on the other hand, was only about five times less

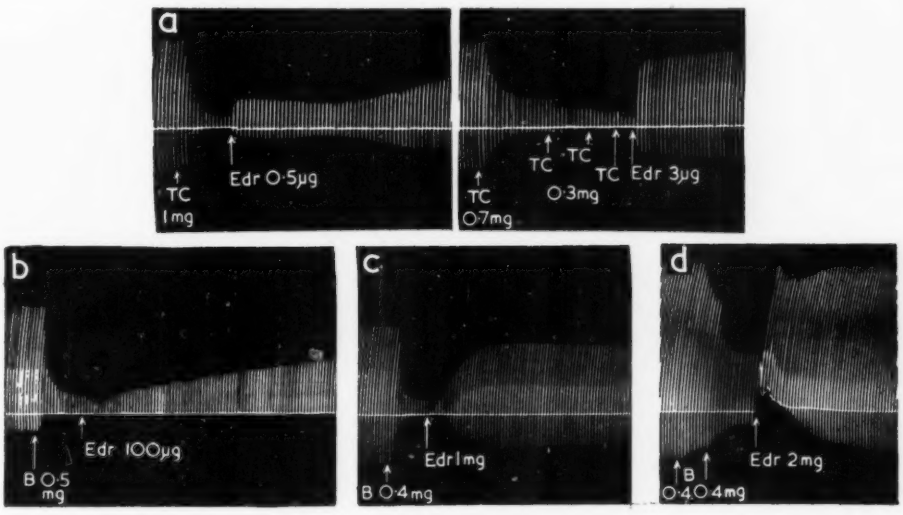

FIG. 3.—a, Cat 3.7 kg.; b, cat 3.2 kg.; c, cat 3.6 kg.; d, cat 4.1 kg. Maximal twitches of the tibialis anterior muscle were elicited indirectly once every 10 sec. At TC, tubocurarine, and at B, benzoquinonium, was injected intravenously. At Edr, edrophonium was injected close-arterially.

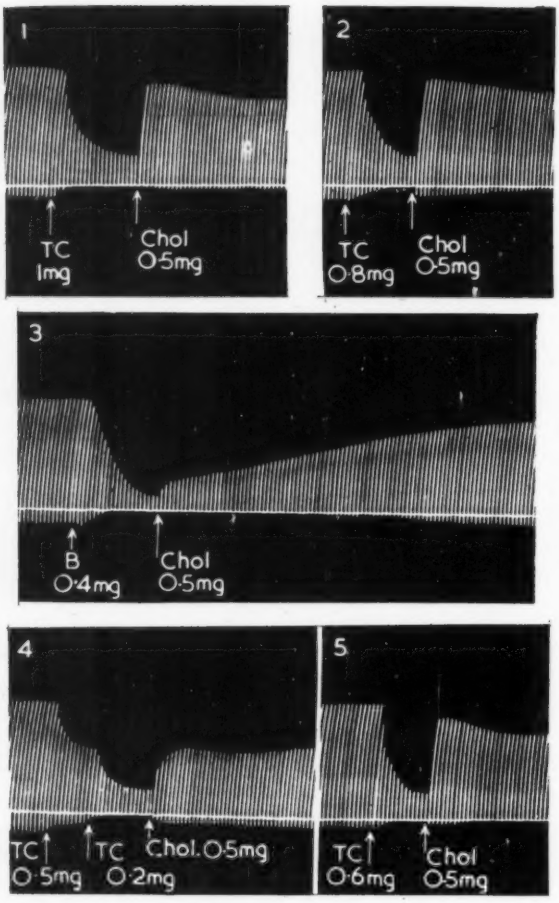

FIG. 4.—Cat 3.4 kg. Maximal twitches of the tibialis anterior muscle were elicited indirectly once every 10 sec. At **TC**, tubocurarine, and at **B**, benzoquinonium was injected intravenously. 45 min. elapsed between injections of the blocking agents. At **Chol**, choline was injected close-arterially.

effective against benzoquinonium than against tubocurarine and its anti-curare effect was never completely abolished after benzoquinonium.

In contrast to edrophonium and choline, tetanic stimulation or the administration of acetylcholine, suxamethonium, or decamethonium was more effective in overcoming paralysis produced by benzoquinonium than by tubocurarine (Fig. 5). These results emphasize again the difference between the actions of choline and edrophonium on the one hand, and those of the purely depolarizing substances on the other. Small doses of acetylcholine were much more effective against benzoquinonium paralysis than against tubocurarine paralysis (Fig. 5), the minimal effective doses being of the order of 15 μg. and 80 μg. respectively. This difference may partly be accounted for by the anticholinesterase action of benzoquinonium (Hoppe, 1951) which, by preventing the hydrolysis of acetylcholine in the blood stream, will allow a greater quantity of that injected to reach the motor end-plates. Large doses of acetylcholine were slightly less effective against benzoquinonium than against tubocurarine (Fig. 5d), possibly because the antagonistic action of large doses of acetylcholine is mainly due to choline itself. Since this latter compound is relatively weak in antagonizing benzoquinonium, this result is not surprising.

Interaction with Injected Acetylcholine

In experiments to study the effects of choline and edrophonium on the response of the muscle to acetylcholine, physostigmine was used for comparison. Maximal twitches were elicited by motor nerve stimulation once every 10 sec. At intervals electrical stimulation was temporarily stopped, and a constant amount of acetylcholine, sufficient to cause a submaximal contraction, was administered close-arterially. After three or four control responses, edrophonium, choline, or physostigmine, in doses known to be sufficient to cause an increase in twitch tension, was administered intravenously. A typical experiment with edrophonium and physostigmine is illustrated in Fig. 6a. While physostigmine always potentiated the response to acetylcholine, edrophonium and choline did not affect it or even slightly depressed it. After physostigmine, the increase in twitch tension produced by edrophonium and choline was always greater, but in spite of this the acetylcholine response was still unaffected.

In a further series of experiments, a similar procedure was carried out except that a partial paralysis of the indirectly excited maximal twitches was maintained throughout by a continuous intravenous infusion of tubocurarine. In the experiment illustrated by Fig. 6b, the amount of acetylcholine had to be increased more than ten-fold during the tubocurarine infusion in order to obtain a response. Edrophonium, injected intravenously, caused an almost complete, though temporary, return of the twitch tension to normal. It did not, however, increase the response to injected acetylcholine. Physostigmine, on the other hand, more than doubled the acetylcholine response. Choline gave similar results to edrophonium.

Synergism with Decamethonium

In addition to having an anticholinesterase action, physostigmine and neostigmine have been

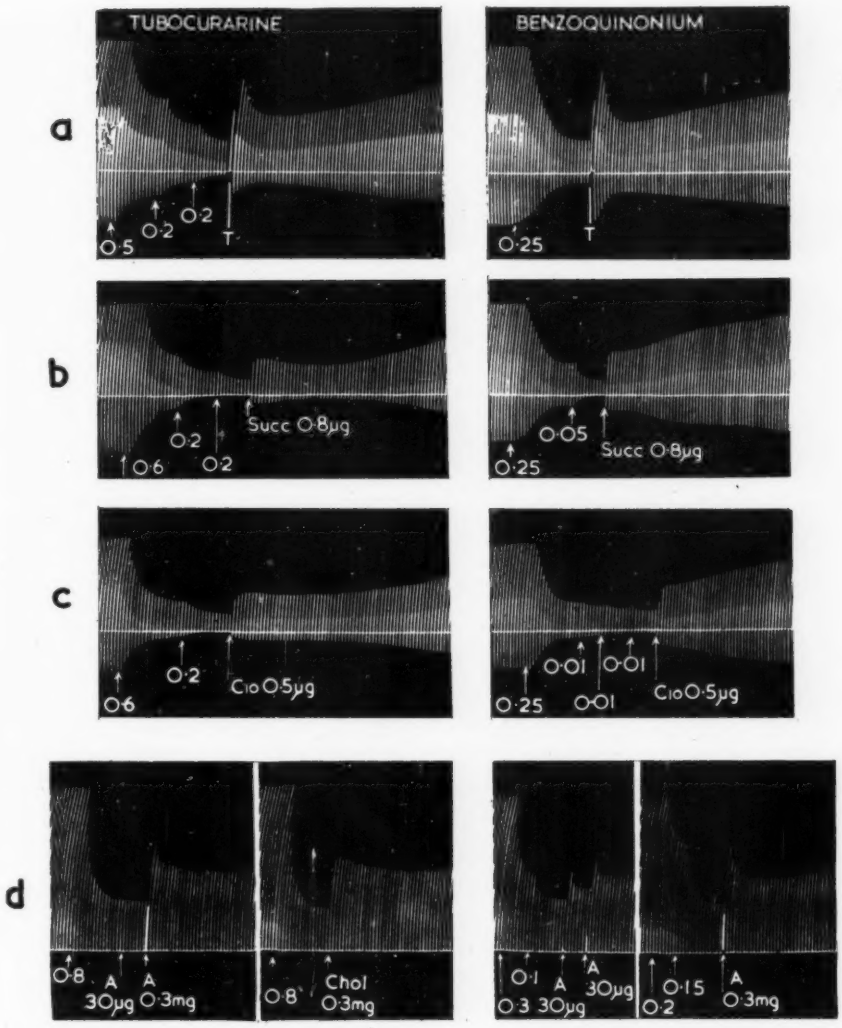

FIG. 5.—**a**, Cat 3.8 kg.; **b**, cat 3.1 kg.; **c**, cat 3.9 kg.; **d**, cat 4.3 kg. Maximal twitches of the tibialis anterior muscles were elicited indirectly once every 10 sec. Records on the left are of paralysis produced by tubocurarine and on the right by benzoquinonium. The unlabelled numerals denote the doses of the blocking agents in mg. administered intravenously. At **T**, the sciatic nerve was stimulated at a frequency of 50/sec. for 4 sec. At **Succ**, suxamethonium, at **C_{10}**, decamethonium, at **A**, acetylcholine, and at **Chol**, choline were administered close-arterially.

shown to potentiate the effects of stable choline esters and other quaternary ammonium bases, apparently by increasing non-specifically the excitability of the effector cells (Brown and Harvey, 1941; Zaimis, 1951; Cohen and Posthumus, 1955). In the present study the doses of edrophonium and choline used did not, as already described, potentiate the response to injected acetylcholine. These results provide evidence that the effects of edrophonium and choline cannot be due to an increase in the excitability of the motor end-plates. Nevertheless, some synergism was shown to exist between choline and edrophonium on the one hand, and decamethonium on the other, for the previous administration of a subthreshold amount of either of the former compounds increased the stimulant action of decamethonium. This synergism, however, need not be a consequence of a sensitization of the motor end-plates by edrophonium and choline; it might simply be an additive effect either between decamethonium and acetylcholine, accumulating in the presence of edrophonium, or between choline and

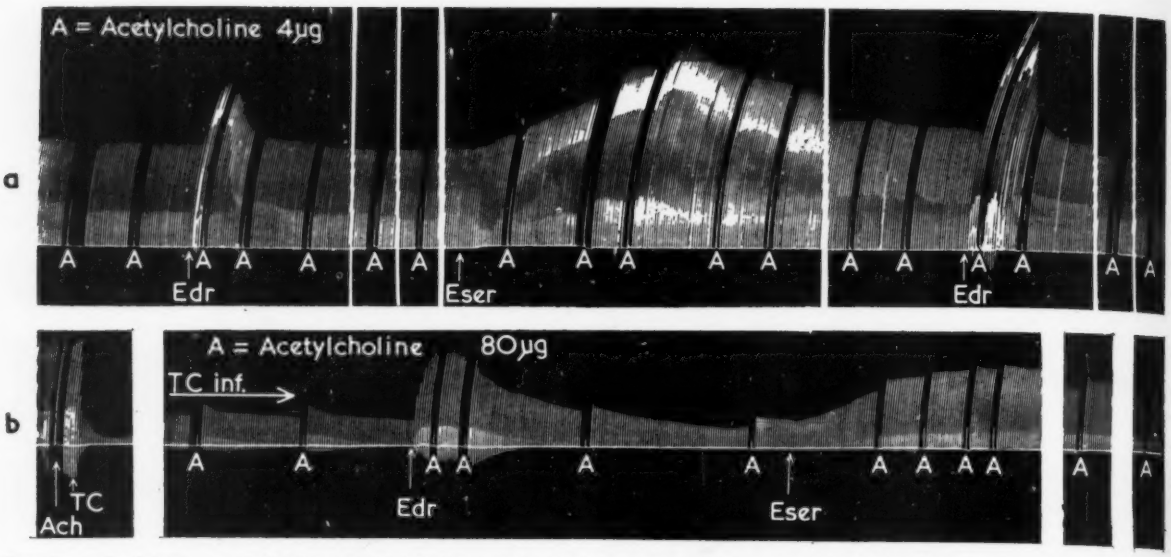

FIG. 6.—**a**, Cat 3.9 kg.; **b**, cat 3.5 kg. Maximal twitches of the tibialis anterior muscles were elicited indirectly once every 10 sec. At **Edr**, 0.5 mg. of edrophonium, at **Eser**, 1 mg. of physostigmine, at **TC**, 1 mg. of tubocurarine, and at **Ach**, 8 μg. acetylcholine were given. The injections were made intravenously, except those of acetylcholine, which were administered close-arterially. In **b**, during the recovery from the effect of the single injection of tubocurarine, a continuous intravenous infusion of tubocurarine (1.7 mg./hr.) was started and maintained throughout the rest of the experiment.

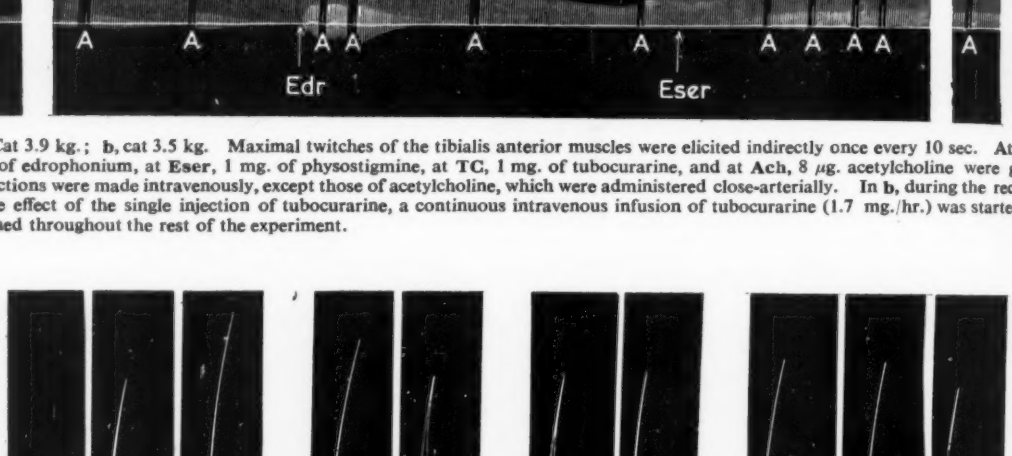

FIG. 7.—Cat 3.7 kg. Chronically denervated tibialis anterior muscle (19 days). At C_{10}, decamethonium, and at **Edr**, edrophonium were injected close-arterially. **C** and **E** were selected as the control doses of decamethonium and edrophonium respectively.

decamethonium, both compounds possessing a direct action on the motor end-plates. Experiments were therefore carried out on chronically denervated muscles from which acetylcholine has disappeared, to determine whether the synergism was due to sensitization of the effector cell. The method was based on that of Gaddum (1959). The quantity of decamethonium necessary to produce a contracture approximately equivalent in tension to 50% of the maximum was determined. The dose, either of edrophonium or of choline, necessary to reproduce this effect was then found, and finally mixtures containing the following proportions of these control doses were prepared: decamethonium : choline or edrophonium; 50% : 50%, 25% : 75%, 75% : 25%. These mixtures were then injected and the responses compared with those elicited by the control doses of each compound alone. Responses to the mixtures similar to those of the control doses would indicate a simple additive effect between the compounds while markedly greater responses would indicate true potentiation.

The responses to mixtures of the compounds containing 50% or more of the control dose of decamethonium showed only a slight increase

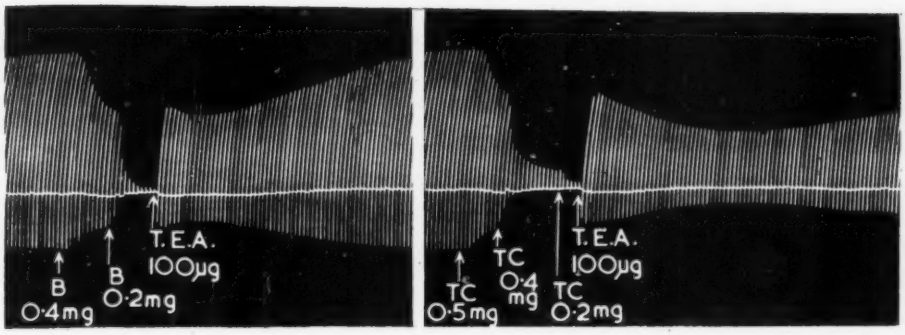

FIG. 8.—Cat 3.9 kg. Maximal twitches of the tibialis anterior muscle were elicited indirectly once every 10 sec. At B, benzoquinonium, and at TC, tubocurarine were injected intravenously. At T.E.A., tetraethylammonium was injected close-arterially.

over the responses elicited by each substance alone, while mixtures containing only 25% often had slightly less action than the control. Fig. 7 illustrates a typical experiment with edrophonium and decamethonium. Synergism between these compounds in the denervated muscle was therefore mainly an additive effect, any true potentiating action being very weak. These results further support the conclusion that an increase in the excitability of the effector cell is unlikely to play a major part in the effects of edrophonium and choline in the skeletal muscles of the cat.

Action of Tetraethylammonium

Stovner (1957a, b, and c ; 1958a, b, and c) and Koketsu (1958) have studied the striking anticurare effect of the tetraethylammonium ion, and concluded that it possesses a pre-synaptic action through which the amount of acetylcholine liberated by each nerve impulse is increased. Because of this, the action of tetraethylammonium on blockade produced by benzoquinonium was studied. Tetraethylammonium was found to be at least as effective in antagonizing paralysis produced by benzoquinonium as that produced by tubocurarine (Fig. 8) showing that its action differs from that of edrophonium and choline. Thus, while all three substances may increase the amount of transmitter liberated by the nerve, there must be some difference in the way in which edrophonium and choline on the one hand, and tetraethylammonium on the other, initiate this effect. Stovner (personal communication) has recently reached the same conclusion.

DISCUSSION

All the effects produced by decamethonium and suxamethonium at the neuromuscular junction are apparently due to a single mechanism of action, namely depolarization of the motor end-plates. For this reason there is little difference between the doses necessary to cause fasciculations of resting muscle, potentiation of the maximal twitch or antagonism to tubocurarine. In the present experiments it was found that the range of doses which produce only potentiation of the maximal twitch, without subsequent blockade, was narrow, and that doses large enough to cause an immediate contraction of the resting muscle were always followed by neuromuscular block.

Edrophonium, on the other hand, caused potentiation of the maximal twitch and antagonism to tubocurarine in doses considerably smaller than those necessary to cause fasciculations of the resting muscle, and the range of doses over which only potentiation of the maximal twitch occurred was extremely wide. Furthermore, even in doses big enough to cause an immediate contraction of the resting muscle, edrophonium did not cause neuromuscular block and the response was usually followed by powerful fasciculations. Extremely large doses were needed to produce neuromuscular block. These results suggest that the direct action of edrophonium on the motor end-plates is very weak and that its effects, except when very large doses are used, must be mediated mainly through acetylcholine. Further evidence that the direct depolarizing action of edrophonium is very weak was obtained from the experiments from benzoquinonium. Acetylcholine, decamethonium, and suxamethonium were effective in antagonizing paralysis produced by benzoquinonium or by tubocurarine, whereas edrophonium antagonized benzoquinonium only in doses at least 300 times greater than those necessary to antagonize tubocurarine. These results agree with those of other workers (Hobbiger, 1952 ; Smith *et al.*,

1952; Nastuk and Alexander, 1954), who have shown that direct end-plate stimulation plays little, if any, part in the anti-curare action of edrophonium.

The effect of choline appeared to be a combination of an acetylcholine-like action at the motor end-plate and of an edrophonium-like action. It resembled decamethonium and suxamethonium in the normally innervated non-curarized muscle, but its ability to antagonize a tubocurarine paralysis was about five times more powerful than would be expected if the effect were entirely due to a direct action.

Similarity between the actions of edrophonium and choline is apparent also from experiments on chronically denervated muscle. The chronically denervated tibialis anterior muscle gave a contracture-like response to both edrophonium and choline. However, the increase in sensitivity of the denervated muscle to these substances was found to be considerably greater than that to both decamethonium and suxamethonium. This is a puzzling result taking into consideration the weak depolarizing action of edrophonium and choline, but two explanations may be suggested. The response of the denervated muscle to these two substances may not be a true contracture like that produced by acetylcholine and similar drugs (Brown, 1937; Zaimis, 1951). For example, adrenaline has been shown to increase the tension of chronically denervated muscles (Bülbring and Burn, 1936), but, during a study of its effects in various skeletal muscles, it was found that the increase in tension of the denervated muscle due to adrenaline is accompanied by electrical events different from those accompanying the contracture produced by depolarizing drugs (Bowman and Zaimis, unpublished observation). Thus edrophonium and choline may have a different action from acetylcholine in denervated muscle. An alternative explanation might be that degeneration of the motor nerve following denervation results in the destruction of receptor sites to edrophonium and choline. Thus, after denervation, more molecules may be available for an action on the muscle fibre itself.

In the present experiments, choline was shown to be about five times less effective as an antagonist to paralysis produced by benzoquinonium than to that produced by tubocurarine. Furthermore, the previous administration of benzoquinonium was shown to reduce the ability of choline to antagonize a blockade produced by tubocurarine. It was previously shown (Bowman, 1958) that benzoquinonium can completely abolish the anti-curare actions of edrophonium, neostigmine, physostigmine, and ethyl pyrophosphate. These results suggest that a similar mechanism of action may account for the effects of the last four compounds and for part of that of choline.

Masland and Wigton (1940) and Boyd and Martin (1956) have shown that, in addition to its ability to inhibit cholinesterase, neostigmine possesses an action on the motor nerve endings. More recently Riker *et al.* (1957, 1959) concluded that an action on the nerve endings, through which neuromuscular transmission is facilitated, is the main mechanism of action of compounds of the edrophonium type. Hutter (1952a) suggested a similar mechanism of action for part of the effect of choline. An action on the motor nerve endings through which the amount of acetylcholine released by a nerve impulse is increased would explain the present results obtained with edrophonium as well as those previously obtained with neostigmine, physostigmine, and ethyl pyrophosphate. It would also account for part of the action of choline. That the previous administration of benzoquinonium prevented the anti-curare action of edrophonium, neostigmine, physostigmine, and ethyl pyrophosphate, and reduced that of choline, could be explained if, as previously suggested (Bowman, 1958), benzoquinonium prevents the action of these substances on the motor nerve endings. This effect of benzoquinonium must be independent of its curare-like effect on the motor end-plate, firstly because tubocurarine and other curare-like substances do not produce it, and secondly because substances stimulating the motor end-plate directly are still active in the presence of benzoquinonium. Benzoquinonium therefore shows promise as a useful pharmacological tool for distinguishing compounds with an edrophonium-like action. It does not prevent the anti-curare action of substances which act by direct stimulation of the motor end-plates; nor does it affect that of the tetraethylammonium ion.

Both *in vitro* and *in vivo* edrophonium possesses only a very weak anticholinesterase action (Randall and Lehmann, 1950; Randall, 1950; Macfarlane, Pelikan and Unna, 1950; Hobbiger, 1952). Nevertheless, in frog muscle this substance potentiates the effects of acetylcholine (Smith *et al.*, 1952; Hobbiger, 1952; Cohen and Posthumus, 1955; Katz and Thesleff, 1957). Such an action appears unlikely to play a major part in the effects of edrophonium in the cat for, in the present experiments, doses which strongly potentiated the maximal twitch or antagonized tubocurarine did not potentiate the

response to injected acetylcholine. Similarly, choline was shown to have no potentiating action on the response to acetylcholine.

In a previous paper (Bowman, 1958) experiments were described in which an initial dose of tubocurarine, sufficient to produce an 80 to 90% paralysis of the indirectly excited maximal twitches, had been administered intravenously. Four hours later edrophonium, neostigmine, physostigmine, or ethyl pyrophosphate had been administered in a dose large enough to produce a marked increase in twitch tension. During this potentiation, either the same dose of tubocurarine or an equipotent dose of benzoquinonium had been administered. Under these conditions the effect of the second injection of tubocurarine had been much reduced, but that of the benzoquinonium was unaffected. This was so despite the fact that acetylcholine is a more effective antagonist to benzoquinonium than to tubocurarine. Since the anti-curare substance had been administered before the benzoquinonium, such experiments ruled out the possibility that the lack of antagonism to benzoquinonium had been due to its preventing, in some way, the combination between anti-curare substance and cholinesterase. These results, therefore, suggested that the increase in twitch tension and the antagonism to tubocurarine produced by these compounds is not primarily a consequence of an accumulation of acetylcholine, for if this were the explanation the blocking effect of benzoquinonium should also have been much reduced. It is an established fact that neostigmine, physostigmine, and ethyl pyrophosphate inhibit the cholinesterase at the neuromuscular junction, but from the present results it seems that the small amounts of acetylcholine that are released, either spontaneously or as the result of a single nerve impulse delivered every 10 sec., even though protected from destruction, must simply diffuse rapidly away from the end-plate region. It would appear, therefore, that an anticholinesterase action cannot by itself account for the antagonistic action of a substance to paralysis produced by tubocurarine. Once the output of acetylcholine is increased by the effect of the drug on the motor nerve endings, any additional ability of the compound to inhibit cholinesterase may become important in the non-curarized muscle, for, by preserving the acetylcholine beyond the refractory period of the muscle fibre, sufficient may remain in the region of the motor end-plates long enough to facilitate repetitive firing.

The main effect of the anticholinesterase drugs, of edrophonium and part of that of choline at the neuromuscular junction appears, therefore, very similar to that following an indirectly elicited tetanus. Several workers (Hutter, 1952b; Liley and North, 1953; Del Castillo and Katz, 1954; Liley, 1956a and b) have presented evidence that, following high frequency stimulation of the motor nerve, both the spontaneous release of acetylcholine and that liberated by single shocks is increased. The results of Hutter (1952a) with choline led him to suggest (Hutter, 1952b) that the increase in acetylcholine output following a tetanus might be a consequence of the action of choline accumulated at the neuromuscular junction during the repetitive stimulation. Choline, however, is a very inactive compound, and it appears unlikely that the local concentration would ever reach a level sufficient to produce such an effect. Furthermore, the present results showed that, while a tetanus was slightly more effective in antagonizing benzoquinonium than tubocurarine, choline was much less effective against the former blocking agent.

Riker *et al.* (1957) concluded from their results that not only the action of edrophonium but also that of acetylcholine itself is mainly on the motor nerve endings. According to them, on the arrival of an impulse at the nerve ending "acetylcholine is released from a bound state in the motor nerve terminal and reacts with pre-synaptic receptors to initiate the transmitter effect." The present results, however, may be adequately explained on the basis of the classical theory of chemical transmission. Furthermore, the results rule out the possibility that edrophonium and acetylcholine have the same site of action since acetylcholine was very effective in antagonizing benzoquinonium paralysis, while edrophonium was virtually ineffective.

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THE SCHISTOSOMICIDAL AND TOXIC EFFECTS OF SOME *N*-*p*-AMINOPHENOXYALKYLAMIDES

BY

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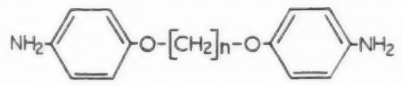
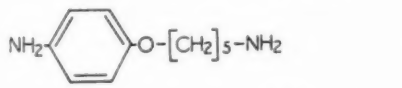
Several *N*-(*ω*-*p*-aminophenoxyalkyl)amides were active against *Schistosoma mansoni* in mice. One of the most effective, *N*-(5-*p*-aminophenoxypentyl)benzamide (M&B3002), acted more rapidly than lucanthone on adult worms but less rapidly than antimony potassium tartrate. It was inactive against immature worms. This compound and M&B2948A (*N*-(5-*p*-aminophenoxypentyl)phthalimide) were both active against *S. mansoni* in hamsters. In monkeys M&B2948A was inactive, whilst M&B3002 was not tested for therapeutic activity. Several of the compounds were examined for the production of visual impairment in cats. Although this property was not entirely absent, its incidence was very much lower in this amide series than among other *ω*-*p*-aminophenoxyalkyl derivatives not containing an amide group. M&B3002 and M&B2948A produced impairment of vision in only a small proportion of the large number of cats tested. The general toxicology of the two drugs was studied in several species, and also their absorption and excretion in mice and rats; this was to provide information for a clinical trial.

Following the discovery that a number of *ωω*-di(*p*-aminophenoxy)alkanes (I) and related monoamines (Raison and Standen, 1955; Caldwell and Standen, 1956; Collins, Davis, Edge, and Hill, 1958) were active against *Schistosoma mansoni* in mice, we prepared and tested other *ω*-*p*-aminophenoxyalkyl derivatives, one of which was the dimethanesulphonate of 5-*p*-aminophenoxypentylamine (1-*p*-aminophenoxy-5-aminopentane) (II). This compound and (5-*p*-aminophenoxypentyl)-dimethylamine and -cyclohexylamine were inactive against *S. mansoni* and free from retinotoxic effects, but the intermediate for the preparation of 5-*p*-aminophenoxypentylamine, namely *N*-(5-*p*-aminophenoxypentyl)phthalimide, (M&B2948A) (III), showed marked activity.



(I)



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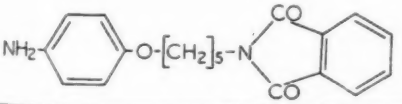

(III)

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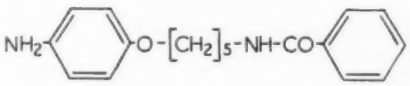
The corresponding benzamide (M&B3002) (IV) was then prepared and found to be active. Many other amides and imides were made subsequently, and this series forms the subject of the present paper.



(IV)

As in the previous investigation (Collins *et al.*, 1958) some of these compounds were examined for retinotoxicity in cats; this is known to be a potential hazard with substances of this type (Edge, Mason, Wien, and Ashton, 1956; Ashton, 1957; Goodwin, Richards, and Udall, 1957; Sorsby and Nakajima, 1958). Although some effect was found in a few of the cats given M&B2948A or M&B3002, it was much less common among the amides and imides than among the earlier compounds. The former were therefore potentially more useful and safer for administration to man, and preliminary clinical trials carried out by Alves, Harper, and Hill (unpublished observations) showed that M&B2948A, when given to children with *S. haematobium* infections, was effective and had no deleterious action on the eyes.

METHODS

Schistosomicidal Activity

The methods employed have been fully described by Hill (1956) and Collins *et al.* (1958). Groups of 12 to 15 mice infected with adult *S. mansoni* were given oral doses daily for four days. They were killed and examined for worms one to two weeks after the end of treatment. In the preliminary screening, test compounds were considered to be active if the number of living worms recovered from the mesenteric veins, portal vein, and liver was significantly fewer (t test, $P<0.01$) than in untreated controls. Many of the active compounds were examined further, using groups of 12 to 15 mice for each dose, to determine the CD50. The LD50 after a single dose was found for each compound, using five mice with each dose, and some of the active drugs were examined further for chronic toxicity by giving four daily doses to groups of 10 mice.

Two of the compounds (M&B2948A and M&B3002) were investigated in more detail than the others. With M&B3002 various dosing schedules were studied, as were the rate of action (Hill, 1956) and the effect on different developmental stages of the worm. Both compounds were also tested for activity against *S. mansoni* in hamsters, and M&B2948A in monkeys. In all these experiments the compounds were given by mouth.

Toxic Effects

Several of the compounds were administered to cats to see if they produced the weakening of the light and blink reflexes which are early signs of visual impairment (Collins *et al.*, 1958). Usually each compound was given to two cats in single oral doses of about 0.4 g./kg. of body weight, but three of the substances were administered to a larger number of cats using higher single and/or multiple doses. The general toxicology of M&B2948A and M&B3002 was examined in several species, the compounds being given orally unless otherwise stated.

Biochemical Estimations

Calibration curves were constructed by adding known quantities of the compounds to blood, urine, and faeces, and assaying the drug.

The methods of estimation all depend on the diazotization of the aromatic amino group of the compounds, followed by reaction with the coupling reagent, *N*-β-sulphatoethyl-*m*-toluidine (Rose and Bevan, 1944). This reagent produced azo dyes with the diazotized compounds with an absorption maximum at 520 mμ. After diazotization and coupling, a few ml. of absolute ethanol were added to the tubes to completely dissolve the azo dyes.

Drug concentrations were estimated as "free" and "total" material. "Free" drug represents diazotizable material prior to hydrolysis whilst "total" drug is that present after hydrolysis. It was always necessary to include "blanks" from biological material containing no drug.

Blood.—Concentration of free drug in blood samples was estimated in protein-free filtrates whereas total drug was estimated in similar filtrates after acid hydrolysis.

Urine.—Free drug was estimated by suitably diluting urine with dilute HCl and diazotizing without further treatment. Similarly, total drug was estimated after acid hydrolysis.

Faeces.—Extracts of faeces were prepared either by grinding with sand or by maceration in the presence of a warm aqueous solution (15%) of trichloracetic acid. The extract was filtered through a pad of Supercel in a small Buchner funnel. Drug estimations were carried out on the clear filtrate as described for urine.

RESULTS

Screening Experiments

Schistosomicidal Activity.—Among the aliphatic amides listed in Table I, the most active were the trichloracetamide (M&B4433) and the *p*-chlorophenoxyacetamide derivatives (M&B3435). The former was more toxic to mice and the latter was not more active than the benzamide derivative (M&B3002, Table II). The hippuramide (M&B3637) was less effective than these two compounds, and it had a very low cumulative toxicity to mice.

M&B3002 was one of the most potent of the benzamides (Table II). Substitution in the amino group of M&B3002 (Table III), other than by a single methyl group, decreased activity.

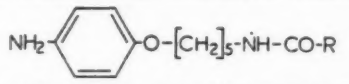
Table IV gives the results with a homologous series of phthalimides. The pentane to the nonane members were effective, although the last was much less so than the others. Isomers of M&B2948A in which the amino group was *ortho* or *meta* to the ether link were inactive, as was also an analogue which had no amino group. Substitution in the amino group of M&B2948A (Table V) reduced activity in all the types examined with the exception of M&B3430.

All the compounds in Tables VI and VII were less active than M&B2948A with the exception of the succinimide (M&B3023), the thenoylamide (M&B3124) and the piperidone (M&B3414), each of which was, however, more toxic than M&B2948A.

Ocular Effects.—None of the compounds produced ocular effects during the preliminary screening test, but M&B2948A, M&B3002, and M&B3637 affected the retinae of a few cats when more extensive examinations were carried out. M&B3012 (Table I) caused shivering, salivation, and repeated vomiting in cats.

TABLE I

ALIPHATIC AND SUBSTITUTED ALIPHATIC AMIDES

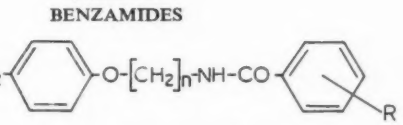
Group formula: 

All compounds were tested as the base except those marked with an asterisk, which were tested as the methanesulphonate. †, positive ocular response in only a few cats (Table VIII); ‡, no ocular response in cats. The following compounds were also therapeutically inactive: *N*-5-*p*-aminophenoxypentyl-*N*-phenylacetamide and 1-(5-*p*-aminophenoxypentyl)-3-*p*-ethoxyphenylthiourea.

M&B No.	–R	LD50 (g./kg.) Single Dose	LD50 (g./kg.) Daily Dose	CD50 Daily Dose (g./kg.)
4434	–H	0·75		>0·20
3012	$-CH_3$	1·5	0·80	0·42‡
4319	$-C_2H_5$	1·0		>0·40
4312	$-[CH_2]_4-CH_3$	>4·0		Inactive
4253	$-CHCl_2$	1·2		0·40
4433	$-CCl_3$	0·50		0·20
4454	$-CH_2CN$	2·5		<0·80
3180	$-CH_2-C_6H_5$	1·0		>0·30
3236	$-[CH_2]_4-C_6H_5$	4·0		Inactive
3208	$-CH=CH-C_6H_5$	>4·0		>1·0
4410	$-CH(C_6H_5)_2$	>4·0		<1·0
3386	$-CH_2-O-C_6H_5$	2·0		>0·60
3435	$-CH_2-O-C_6H_4-Cl-p$	>4·0		0·20
3584	$-CH_2-O-C_6H_3-Cl_2-2,4$	>4·0		>0·20
3751	$(\pm)-[CH_2]_2-NH-CO-CH(OH)-C(CH_3)_2-CH_2OH$	>1·0		>0·50
3637	$-CH_2-NH-CO-C_6H_5$	>4·0	3·0	0·38†
3707	Phthalimidomethyl	>4·0		>1·0
3237	Cyclohexyl	>4·0		Inactive
3169	$-OC_2H_5$*	1·0		>0·3
4448	$-CO-OC_2H_5$*	0·75		Inactive
3166	$-NH_2$	1·5		,,
4381	$-NH-[CH_2]_5-O-C_6H_4-NH_2-p$	>4·0		,,
4349	$-CO-NH-[CH_2]_5-O-C_6H_4-NH_2-p$	>4·0		>1·0
3241	$-[CH_2]_3-CO-NH-[CH_2]_5-O-C_6H_4-NH_2-p$*	3·0		Inactive

TABLE II

BENZAMIDES

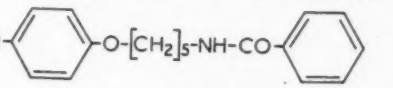
Group formula: 

All compounds were tested as the base except that marked with an asterisk, which was tested as the methanesulphonate. †, Positive ocular response in only a few cats (Table VIII); ‡, no ocular response in cats. *N*-(5-*p*-aminophenoxy-1-phenylpentyl)benzamide was also therapeutically inactive.

M&B No.	n	–R	LD50 (g./kg.) Single Dose	LD50 (g./kg.) Daily Dose	CD50 Daily Dose (g./kg.)
3210	4	–H	3·0		Inactive
3002	5	–H	>4·0	0·88	0·20†
3189	8	–H	4·0	0·57	0·16‡
4536	5	$p-NO_2$	>4·0		<1·0
3104	5	$p-NH_2$*	1·0		Inactive
3694	5	$p-NH-CO-CH_3$	>4·0		>0·20
3196	5	p-Cl	>4·0		>1·0
4422	5	p-Br	>4·0		1·0
3194	5	$p-CH_3$	>4·0		Inactive
3629	5	o-OH	>4·0		,,
3541	5	p-OH	1·0		,,
3487	5	$o-OCH_3$	3·0		>0·20
3439	5	$m-OCH_3$	1·5		0·20
3161	5	$p-OCH_3$	>4·0	0·69	0·29‡
3373	5	$o-CO_2Li$	3·0		>0·80
4308	5	$p-CO_2H$	>4·0		Inactive
4260	5	$p-CO_2CH_3$	1·5		,,

TABLE III

BENZAMIDES, SUBSTITUTED IN THE AMINO GROUP

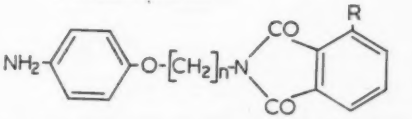
Group formula: 

All compounds were tested as the base.

M&B No.	–R	LD50 (g./kg.) Single Dose	LD50 (g./kg.) Daily Dose	CD50 Daily Dose (g./kg.)
3670	$-NHCH_3$	>4·0	1·0	0·20
3300	$-N(CH_3)_2$	>4·0	0·83	0·50
3556	$-NH-CH_2-CH_2OH$	4·0		0·40
3199	$-N(CH_2-CH_2OH)_2$	>4·0	1·8	>0·80
3572	$-NH-CH_2-CH_2-OCH_3$	2·0		>0·60
3549	$-NH-CH_2-CH(OH)-CH_2OH$	>4·0		>1·0
3552	$-N[CH_2-CH(OH)-CH_2OH]_2$	>4·0		Inactive
3561	$-N[CH_2-CH(OH)-CH_3]_2$	>4·0		<1·0
3380	$-NH-CH_2-CO-NH_2$	>4·0		Inactive
3416	$-NH-CH_2-SO_3Na$	4·0		>0·20
3385	$-NH-CO-CH_3$	>4·0		Inactive
3734	D-Glucosylamino	>4·0		>0·40

TABLE IV

PHTHALIMIDES

Group formula: 

All compounds were tested as the base except those marked with an asterisk, which were tested as the methanesulphonate. †, Positive ocular response in only a few cats (Table VIII); ‡, the corresponding hex-3-ene, tested as the methanesulphonate, had an LD50 (daily dose) of 1·0 mg./g. and a CD50 >0·20 mg./g. The following compounds were also therapeutically inactive: *N*-(5-*p*-aminophenoxy-3-methylpentyl)phthalimide, *N*-(5-*p*-aminophenoxy-1-phenylpentyl)phthalimide and *N*-(5-*p*-aminophenoxypentyl)tetrachlorophthalimide.

M&B No.	n	–R	LD50 (g./kg.) Single Dose	LD50 (g./kg.) Daily Dose	CD50 Daily Dose (g./kg.)
3155	2	–H*	0·8		Inactive
3058	3	–H*	1·5		,,
3192	4	–H	>4·0		,,
2948A	5	–H	>4·0	1·02	0·18†
3153	6‡	–H	>4·0	1·24	0·15
3215	7	–H	4·0		0·14
3167	8	–H	>4·0		0·22
3187	9	–H	>4·0		>1·0
3291	10	–H	>4·0		Inactive
3693	5	$-NO_2$	1·5		,,
3752	5	$-NH_2$	>4·0		,,

Selection of Compounds for Further Study

M&B2948A and M&B3002, the first compounds of this type to be tested in mice, showed equal activity to lucanthone, but had lower chronic toxicity.

	CD50 (g./kg./day)	LD50 (g./kg./day)
Lucanthone	0.19	0.18
M&B2948A	0.15	1.20
M&B3002	0.20	0.88

Because of this and since at the time they were thought to be free from the ocular effects associated with some related compounds (Goodwin *et al.*, 1957; Collins *et al.*, 1958), further toxicity and therapeutic experiments were carried out to provide information for a clinical trial.

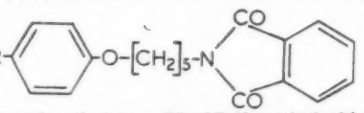
Later, M&B3637 (Table I) was found to be less toxic than M&B2948A and M&B3002 on repeated administration to mice, and, since the last two compounds were then known to produce ocular effects in a few cats, M&B3637 was also given to several cats (Table VIII) to see if it was completely free from these effects. However, no further experiments were done when it was found also to produce mydriasis and especially since it was less active in mice than M&B2948A and M&B3002. The hexane and heptane homologues of M&B2948A (Table IV) were not sufficiently more active than M&B2948A to warrant further study, particularly since the intermediate compounds were slightly less accessible chemically. None of the other compounds showed any advantage over M&B2948A and M&B3002 in the screening tests.

Results with M&B2948A and M&B3002

Schistosomicidal Activity. — Two rhesus monkeys and one green monkey (*Cercopithecus*

TABLE V

PHTHALIMIDES, SUBSTITUTED IN THE AMINO GROUP

Group formula: 

All compounds were tested as the base. The LD50 marked with an asterisk was 0·8 g./kg. when the drug was given in daily doses.

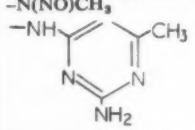
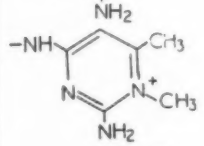
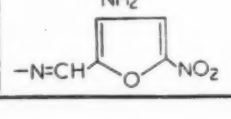
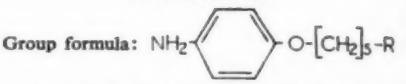
M&B No.	–R	LD50 Single Dose (g./kg.)	CD50 Daily Dose (g./kg.)
3413	$-NHCH_3$	>4·0	>0·20
3430	$-N(CH_3)_2$	4·0	0·23
3031	$-N(CH_2-CH_2OH)_2$	>4·0*	>0·40
4202	$-N(CH_2-CH_2Cl)_2$	>4·0	Inactive
3782	$-NH-CO[CH_2]_2-CO_2NH_4$	4·0	,,
4186	$-N(NO)CH_3$	>4·0	,,
3763		>4·0	>1·0
3819		>4·0	Inactive
4396		>4·0	,,

TABLE VI

OTHER IMIDES

Group formula: 

All compounds were tested as the base except that marked with an asterisk (as the methanesulphonate) and that marked † (as the sulphate). ‡, No ocular response in cats; §, LD50 (daily dose) =0·5 g./kg.

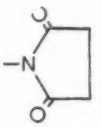
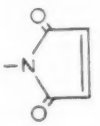
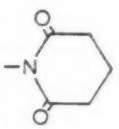
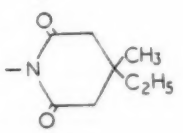
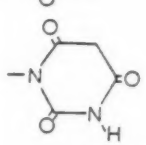
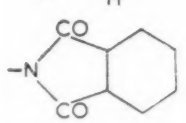
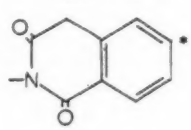
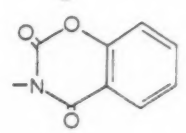
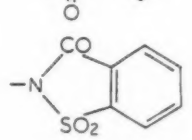
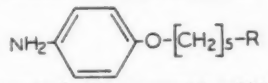
M&B No.	–R	LD50 Single Dose (g./kg.)	CD50 Daily Dose (g./kg.)
3023		3·5§	0·19‡
4310		1·5	>0·40
3885		3·0	>0·40
3952		>4·0	>0·20
3741		>4·0	Inactive
3429		>4·0	0·30
3586		>4·0	>1·0
3461		>4·0	>0·20
3105		>4·0	>0·20‡
4562	(±)–Camphorimido†	4·0	<0·80
3495	$-N(CO-CH_3)_2$	3·0	0·80
3718	$-N(CO-C_6H_5)_2$	>4·0	>1·0

TABLE VII
OTHER AROMATIC CARBOXYAMIDES

Group formula: 

All the compounds were tested as the base except that marked with an asterisk, which was tested as the methanesulphonate.

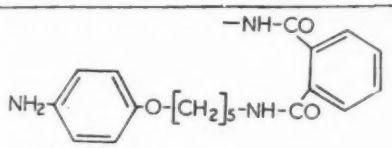
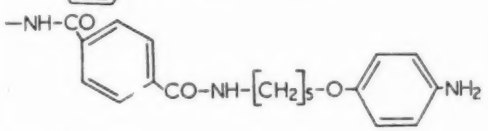
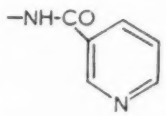
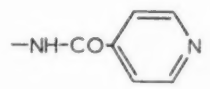
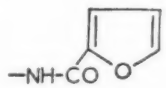
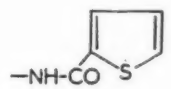
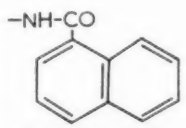
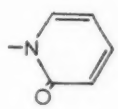
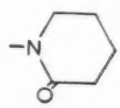
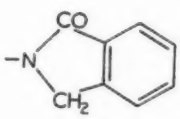
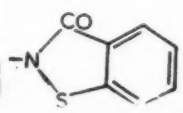
M&B No.	–R	LD50 (g./kg.)		CD50 Daily Dose (g./kg.)
		Single Dose	Daily Dose	
3614		>4·0		>1·0
4262				Inactive
3181		1·0	0·35	>0·20
3207		1·5		>0·40
3212		3·0	0·45	>0·45
3214		3·0	0·28	0·21
3209		>4·0	0·60	0·43
3205	–N(CH_3)–CO–C_6H_5	0·75		>0·25
3137	–N(C_6H_5)–CO–C_6H_5	3·0		>0·80
3160	–N(cyclohexyl)–CO–C_6H_5*	>4·0		Inactive
3630	–N(CO–C_6H_5)–[CH_2]$_5$–O–C_6H_4–NH_2-*p*	1·5		>0·40
3727	–N(OH)–CO–C_6H_5	>4·0		Inactive
3728	–N(O–CH_2–C_6H_5)–CO–C_6H_5	>4·0		0·80
3301		1·5		Inactive
3414		1·5		0·20
4265		>4·0		>0·20
4301		>4·0		Inactive

TABLE VIII

THE OCULAR EFFECTS OF M&B2948A AND M&B3002 IN CATS

The asterisk refers to the methanesulphonate of M&B2948A, *ca.* 0·1% soluble in water; †, the methanesulphonate of M&B3002, *ca.* 3·0% soluble in water; ‡, administered twice daily; §, three of these cats had previously had one dose of either 1·3 or 0·4 g./kg., and had not shown ocular effects; ||, see Table I for formula. Values in brackets refer to numbers of cats.

M&B No.	Daily Oral Dose (g./kg.)	No. of Doses	No. Showing Ocular Effects
2948A	1·0 (10)	3–4	1
	0·8 (2) and 0·4 (2)	1	1 (given 0·4 g./kg.)
	0·5 (13)	3–4	3
	0·25 (10)	3–4	2
	0·125 (10) and 0·08 (10)	4	0
2948*	1·6 (1) and 1·3 (1)	1	0
	0·8 (11)§	1	5
	0·5–0·4 (16)	4–20	3 (1 after 4, 1 after 5, and 1 after 20 doses)
	0·4 (7), 0·25 (2), and 0·15 (2)	1	0
	0·04 (2)	6‡	0
3002	1·9 (1)	1	1
	1·0 (10)	2–4	4
	0·5 (10)	2–4	2
	0·45–0·40 (14)	4–20	5 (2 after 4, 1 after 14,‡ and 2 after 20 doses)
	0·4 (6)	1	1
	0·25 (10), 0·125 (10) and 0·08 (10)	3–4	0
	0·04 (1)	6‡	0
3002A†	0·4 (4)	1	3
	0·04 (1)	6‡	0
3637||	0·8 (8)	3–4	2

sp.) infected with *S. mansoni* were given by mouth 0.4 g./kg. of M&B2948A daily for four days. Two other infected rhesus monkeys were kept as controls. All the treated monkeys continued to pass viable eggs for 23 days after the end of treatment. One of the rhesus monkeys was then killed and examined; the remaining monkeys continued to pass eggs for at least 45 days after treatment. The monkey which was killed had four pairs of worms in the mesenteric veins and one male worm in the liver. No signs of ensheathed worms were found in liver-crush preparations. The drug was inactive, therefore, although similar treatment was highly effective in mice.

M&B2948A was active against *S. mansoni* in hamsters after four daily doses of 0.4 g./kg. This treatment killed a total of 57 out of 60 worms in three hamsters.

With M&B3002, the effect of various dosing schedules was investigated in mice. A single dose, doses once daily for two days, twice daily for two days, once daily for four days, twice daily for four days, and once daily for eight days, giving total doses of 0.8 g./kg., were employed in each instance. There was no significant difference between the effects produced by these treatments.

The rate of action of M&B3002 was studied after a single oral dose of 1.6 g./kg. After 24 hr. the worms had begun to drift back to the liver, although about 20% of them (a total of 25 pairs in 12 mice) were still in the mesenteric veins. The males and females in the portal vein and liver were beginning to separate. After 48 hr. only 3% (a total of 3 pairs in 11 mice) of the schistosomes were found in the mesenteric veins, and 4% in the portal vein. Ensheathment had already begun although none of the worms was completely ensheathed, their ends being free and mobile (Standen, 1955). Seven days after dosing many of them were completely ensheathed. The ratio of males to females on this occasion was 9:1, which may have been due to the more rapid ensheathment of the females or to the fact that they were missed because they had become so small and degenerate. All the females which were seen were paired in the mesenteric veins, but only 11% of the males (a total of three in five mice) were in this site, the rest being in the liver. Fourteen days after treatment the sex ratio was 1:1, as it had been at the beginning of the experiment. Half of the living worms were found in the mesenteric veins and half in the portal vein, but the average number of schistosomes/mouse was only 0.25 pairs in eight mice compared with 19.8 pairs for the 12 untreated controls examined at the beginning of the experiment. About 98% of the worms had, therefore, been killed by the drug.

The effect of M&B3002 was also investigated on worms at various stages of development. Five groups of mice at 4 to 5 weeks, 5 to 6 weeks, 6 to 7 weeks, 7 to 8 weeks, and 8 to 9 weeks respectively after infection were all given 0.4 g./kg. daily for four days. They were examined two weeks after treatment or seven to eight weeks after infection, whichever was the later. The results showed that M&B3002 was inactive against worms 4 to 5 weeks old, and that the activity increased from 5 to 6 to 8 to 9 weeks. Another experiment in which mice were treated daily for five days and infected between the second and third doses demonstrated that M&B3002 had no prophylactic activity.

A dose of 0.4 g./kg. of M&B3002 daily for four days was found, like M&B2948A, to be active against *S. mansoni* in hamsters. A total of 61 out of 66 worms in four hamsters were killed.

General Toxicology.—A single oral dose of 4.0 g./kg. of M&B2948A was not lethal to mice, and 4.0 g./kg. of its methanesulphonate was not lethal to mice, rats, and rabbits. The same dose

of M&B3002 was not lethal to mice and rabbits, but caused some deaths in rats, the LD50 in this species being about 3.0 g./kg. (five rats/dose).

Four daily doses of 1.0 g./kg. of M&B2948A were not lethal to cats and monkeys (Tables VIII and IX) although the LD50 in mice was 1.02 g./kg. (Table IV). Guinea-pigs tolerated four daily doses of 0.4 g./kg., but higher doses were not given.

TABLE IX

THE TOXIC EFFECTS OF M&B2948A AND M&B3002 IN RHESUS MONKEYS

The drug marked by an asterisk was the methanesulphonate of M&B2948A, *ca.* 0·1% soluble in water.

M&B No.	Daily Oral Dose (g./kg.)	No. of Doses	No. of Monkeys Treated	No. Showing Ataxia	No. Showing Ocular Effects
2948A	1·0	4	8	0	0
	0·5	4	8	0	0
2948*	1·0	5 and 20	2	0	0
3002	1·0	4	10	9	0
	0·5	4	10	2	0

Four daily doses of 1.0 g./kg. of M&B3002 were not lethal to cats (Table VIII) although they killed seven out of nine rats. The LD50 in mice was 0.88 g./kg. (Table II) and in guinea-pigs about 0.2 g./kg. (five out of nine being killed by this dose). In monkeys (Table IX), four daily doses of 1.0 g./kg. and 0.5 g./kg. produced ataxia and general weakness so that the affected monkeys had to be killed.

M&B2948A and M&B3002 were administered in the diet for 12 days to young rats (five males and five females/group) weighing 50 to 60 g. (Table X). There was some depression of weight gain with 1% M&B2948A and with 0.4% M&B3002, but growth rates returned to normal as soon as non-medicated diet was substituted. Lucanthone retarded the growth rate at 0.12%, but was without effect at 0.02%.

TABLE X

EFFECT OF M&B2948A AND M&B3002 ON GROWTH RATE IN RATS

The period marked by the asterisk is the treatment period; †, the methanesulphonate of M&B2948A.

Compound	Concentration in Diet (%)	Average Weight Gain (g.)	
		Days 1–12*	Days 13–22
M&B2948A ..	1·0	7·5	30
Lucanthone ..	0·02	34	25
Controls	—	39	25
M&B3002 ..	0·4	5	35
M&B2948† ..	0·4	25	30
Controls	—	32	30
Lucanthone ..	0·12	−1	40
,, ..	0·05	20	35
Controls	—	31	27

Vomiting was observed in 10 out of 57 cats given M&B2948A, in 24 out of 70 given M&B3002 and in 8 out of 11 dogs given the methanesulphonate of M&B2948A.

Two monkeys were given 0.25 g./kg. of the methanesulphonate of M&B3002 intraperitoneally twice daily. One of these was killed on the seventh day of the experiment, having been treated for five days. It had become listless and subdued by the third day and this condition increased in severity until the animal was moribund. No effect on the eyes was observed and no macroscopic abnormalities were seen *post mortem*. The second monkey was treated for only three days when it had to be killed. Signs of toxicity included listlessness, partial paralysis of the limbs, and vomiting. Again no ocular effects were observed.

No abnormalities were seen in the blood pictures or blood urea concentrations of three guinea-pigs given 0.4 g./kg. of M&B2948A or 0.1 g./kg. of M&B3002 five times a week for three weeks. Histological studies carried out by Professor N. Ashton showed, however, that both compounds were capable of producing in guinea-pigs some focal necrosis in the liver and kidneys, and inflammatory changes in the lungs, although the degree of pathological change was variable. The principal feature was the presence of hyaline material in the glomerular capillaries and in the lungs, giving a positive reaction to periodic acid-Schiff reagent for the detection of glycogen.

With M&B3002, death, when observed, was preceded by prostration, and the guinea-pigs exhibited weakness, particularly of the hind limbs; breathing was shallow and the body temperature was 35° or less. The actual cause of death remains unknown.

Ocular Effects in Cats.—Only a small proportion of the cats treated with M&B2948A or M&B3002 showed visual impairment (Table VIII).

The eyes of two cats given 20 doses each of 0.4 g./kg. of the methanesulphonate of M&B2948A and killed 23 days after the last dose were examined histologically by Professor N. Ashton, who found no abnormalities in one cat, and the following picture in one of the eyes of the other animal, the second eye being normal. "Immediately to one side of the disc there is a sharply circumscribed area in which the outer layers of the retina have been destroyed and become gliosed. Elsewhere the retina shows slight rosette formation—but no degeneration or pigmentary disturbance. Optic nerve normal."

A third cat which had been given 14 similar doses and killed 48 days after the end of treatment showed "acute-angle retinal folds in all sections of both eyes. At the base of some of these folds there was a slight proliferation of pigment epithelial cells." Professor Ashton said that this cat appeared "to have recovered from very slight early changes."

A fourth cat was given five daily doses of 0.5 g./kg. of the same compound, and subsequently its behaviour indicated gross impairment of vision. It was killed two days after the last dose. Professor Ashton's report on this cat was: "Right eye: The only abnormality in this eye is to be seen in the retina, which shows almost total destruction of the rod and cone layer with acute-angled folding of the retina in the region of the disc. The pigment layer is normal posteriorly but has proliferated anteriorly to form clumps of cells containing aggregations of pigment. Special staining (PAS, Weils, Holmes, and phosphotungstic stains) shows that the remainder of the retina, ganglion cells, and optic nerve are unaffected. Left eye: Shows an exactly similar picture."

A fifth cat given 20 daily doses of 0.45 g./kg. in the same experiment was killed 106 days after the last dose and the report for both eyes was: "There is a patchy loss of the rod and cone layer but nowhere is the destruction complete. In the affected regions there is considerable pigmentary disturbance and occasional angulation of the retina. Aggregation and migration of pigment are particularly marked at the equator where a few colloid bodies are seen. The inner layers of the retina and optic nerve are normal."

Nothing abnormal was found in the eyes of two cats given 0.04 g./kg. of the methanesulphonate of M&B2948A twice daily for three days.

Professor Ashton also examined the eyes of three cats which had received M&B3002 (0.4 g./kg. × 20; 0.4 g./kg. × 14; 0.04 g./kg. × 6) and of one which had had the methanesulphonate of this base (0.04 g./kg. × 6). He found no changes that could be ascribed to the toxic action of the drug, although one of these animals (M&B3002 0.4 g./kg. × 14) exhibited mydriasis.

Absorption and Excretion.—The results of these studies are given in Tables XI and XII. The values were obtained from pooled samples from 15 mice or 3 rats for blood, and 30 mice or 6 rats for urine and faeces. The blood concentration of M&B2948A in rats rose to 6 μg./ml. at 2 hr., remained there until 4 hr., and was undetectable (less than 1 to 2 μg./ml.) at 6 hr. In both species most of the drug was excreted in the first 24 hr. and very little was recovered after that period.

TABLE XI
BLOOD CONCENTRATIONS OF M&B2948A AND M&B3002
Blood concentrations refer to total drug, with free drug concentrations in brackets.

Animal	M&B No.	Dose (g./kg.)	Blood Concentration (μg./ml.) 1 hr.	4 hr.	8 hr.	24 hr.
Mice	2948A	0·25	8·0 (0)	2·0 (0)	1·5 (0)	0
	3002	0·25	30·5 (11·0)	23·0 (1·0)	9·0 (0)	0
Rats	2948A	0·25	0	6·0 (0)	0	0
	3002	0·25	11·0 (0)	5·0 (3·0)	3·0 (0)	0

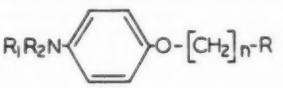
TABLE XII
EXCRETION OF M&B2948A AND M&B3002

Animal	M&B No.	Dose (g./kg.)	Excretion Route	% Dose Excreted (72 hr. Period) Free	Total	Drug Recovery (%)
Mice	2948A	0·25	Urine	2·7	10·0	39·0
			Faeces	22·2	29·0	
	3002	0·25	Urine	5·2	20·6	50·5
			Faeces	28·3	29·9	
Rats	2948A	0·25	Urine	1·3	5·3	30·1
			Faeces	21·9	24·8	
	3002	0·25	Urine	3·1	26·0	46·4
			Faeces	18·0	20·4	

DISCUSSION

Screening Experiments

The active compounds discussed in this report are variants of the general formula which Collins



et al. (1958) considered to contain all the structural features essential for schistosomicidal activity in this chemical field. R_1 and R_2 can be hydrogen, lower alkyl, hydroxyalkyl, or alkoxyalkyl, and R, in addition to substituted or unsubstituted alkoxy, phenoxy, or phenyl moieties, as shown previously, may be an acylamido or imido group, although as pointed out in the introduction the parent amine (II) from which these acyl derivatives were developed was itself inactive. But whereas in the homologous series of diamines previously reported, activity extended from the propane (n=3) to the nonane (n=9) members, in a similar series of phthalimides (Table IV) only the pentane (n=5) to the nonane (n=9) homologues were active. In the above general formula, therefore, n must be 5 to 9 for the imides instead of 3 to 9 as it was for the diamines.

The inactivity of the phthalimides either without an aromatic amino group or with the amino group *ortho* or *meta* to the ether link confirms our previous finding that a *para* amino group is essential for activity.

The incidence of visual impairment in cats among the amides and imides was certainly much lower than it was among the compounds described by Collins *et al.* (1958), although the effect was not absent altogether.

M&B2948A and M&B3002

The inactivity of M&B2948A in monkeys was surprising in view of its performance in mice and hamsters, but this may be due to poor absorption or to a difference of metabolism in the different species, either of which reasons could also explain the lack of toxic signs in monkeys.

The rate of action of M&B3002 resembled that of 1,5-di(*p*-aminophenoxy)pentane dihydrochloride (M&B968A) which was found by Hill (1956) to be more rapid in its action than lucanthone (Miracil D). With the latter compound the worms had only just begun their shift to the liver seven days after treatment, and this migration was only complete after 14 days; also, ensheathment was not noticed until the 21st day, whereas with M&B3002 and M&B968A it was well advanced by the seventh and ninth days respectively. In a hitherto unpublished experiment a single intraperitoneal dose of 0.4 g./kg. of antimony potassium tartrate (tartar emetic) drove all the worms back to the liver within 6 hr., but they recovered and resumed their normal distribution within seven days. The action of antimony potassium tartrate was, therefore, much more rapid than that of the other compounds, but unlike them a single dose exerted only a temporary effect.

The poor activity of M&B3002 against immature worms is paralleled by that of the Miracils (A to D) which was recorded by Kikuth and Gönnert (1948). Bueding, Peters, Koletsky, and Moore (1953) have suggested that on reaching maturity the worms change from an oxidative to a glycolytic way of life; and since they reach maturity at about six weeks after infection, at which time M&B3002 began to be effective, it seemed possible that this compound acted by inhibiting glycolysis. One of us (H. W. R.) has indeed obtained biochemical evidence that compounds of this type do inhibit anaerobic glycolysis in schistosomes.

Toxic Effects.—M&B3002 was more toxic than M&B2948A in rats, guinea-pigs, and monkeys, an observation which in rats at least might be related to the better absorption of M&B3002, although in mice better absorption was accompanied by only a small increase in chronic toxicity which was probably not significant. M&B3002 was more toxic to guinea-pigs than to any of the other species, but it is not possible to say why, unless it was due to better absorption or to a difference in metabolism.

Turning to ocular effects, the methanesulphonate of M&B2948A in particular was shown to be capable of producing the gross destruction of the retina described by Ashton (1957) for 1,5-di(*p*-aminophenoxy)pentane dihydrochloride (M&B968A) although like M&B2948A and M&B3002 it was mydriatic in only a very small proportion of the cats. The methanesulphonate of M&B3002, the only really soluble compound tested, was more retinotoxic than the base, an observation which suggests that the lower incidence among the free bases was connected with their very low solubility, although several of the amines described by Collins *et al.* (1958) were toxic even when administered as insoluble bases.

Neither the benzamide M&B3002 nor the phthalimide M&B2948A produced any ocular effects in monkeys, even at a lethal dose, although 1,5-di(*p*-aminophenoxy)pentane dihydrochloride (M&B968A) was undoubtedly retinotoxic in single non-lethal oral doses of 0.4 g./kg. (unpublished observation). We therefore conclude that the amides and imides discussed here are less retinotoxic in monkeys and cats than the compounds described by Goodwin *et al.* (1957) and Collins *et al.* (1958).

Both M&B2948A and M&B3002 were examined for activity against *S. haematobium* in African schoolchildren, but as no difference could be detected between them in the preliminary trials (Alves and Harper, personal communication), either as regards tolerance or activity, M&B3002 was discontinued since it is slightly less accessible chemically than M&B2948A (Alves, Harper, and Hill, unpublished observations). Schneider and Sansarricq (1959) have found M&B2948A moderately effective against *S. haematobium* infections in Africans.

We wish to thank Professor N. Ashton for carrying out the pathological studies, our colleagues, Dr. J. N. Ashley, Dr. H. J. Barber and Dr. R. Wien, for their helpful discussions during this investigation, Miss N. E. Sirett for the preparation of some of the compounds, Mr. W. A. Freeman for carrying out the blood investigations and Mr. H. S. Alexander, Mr. I. Hodges and Mr. A. D. Speed for their technical assistance.

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THE ANTAGONISM OF COCAINE TO THE ACTION OF CHOLINE 2,6-XYLYL ETHER BROMIDE AT SYMPATHETIC NERVE ENDINGS

BY

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Choline 2,6-xylyl ether bromide (TM10) prevents contraction of the nictitating membrane and of the spleen in response to sympathetic nerve stimulation. It was confirmed that this effect was due to prevention of the release of noradrenaline (" sympathin ") at the nerve endings. The intravenous injection of cocaine before choline xylyl ether blocked the action of the latter drug. However, when atropine was injected before the cocaine the effects of large doses of choline xylyl ether were not completely blocked. After block had been produced by choline xylyl ether the injection of cocaine partially restored both the secretion at the sympathetic nerve endings and the responses of the organs supplied by the nerves. Phenoxybenzamine did not restore the secretion at the nerve endings after it had been inhibited by choline xylyl ether, indicating that the latter did not cause an increased uptake of sympathin by the receptors.

While investigating the pharmacological effects of nuclear substituted choline phenyl ethers, Hey and Willey (1954) noticed that choline 2,6-xylyl ether bromide (TM10; hereafter called choline xylyl ether) blocked the responses of the nictitating membrane of the cat to pre- and post-ganglionic stimulation of the cervical sympathetic nerve. They also showed that choline xylyl ether had a local anaesthetic action almost as powerful as that of cocaine. This led them to suggest that choline xylyl ether might act by suppressing conduction in postganglionic sympathetic nerve fibres. Exley (1957) extended the observations of Hey and Willey (1954) by showing that choline xylyl ether produced a transient ganglionic block, but that it did not prevent the passage of impulses along the nerve fibres. He also showed that the impulses arriving at the nerve endings were in some way rendered ineffective, as noradrenaline did not appear in splenic venous blood when the splenic nerves were stimulated after doses of choline xylyl ether.

While working on the effects of diphtheria toxin on the responses of smooth muscle to various sympathomimetic amines, it was necessary to prevent the secretion of noradrenaline (sympathetic transmitter, " sympathin ") at the ends of the sympathetic nerve fibres. Although choline xylyl ether appeared to be ideally suited for this purpose it was surprising to find that the drug was without effect. Our experimental conditions differed from those of previous workers only in that the animal had been given cocaine. Further experiments showed that cocaine was extremely effective in antagonizing the action of choline xylyl ether, and the results of these experiments are reported here.

METHODS

Operative Procedures.—The experiments were performed on cats anaesthetized with ether followed by chloralose (80 mg./kg. body weight). In all experiments systemic arterial pressure was measured with a mercury manometer The preganglionic fibres of the left cervical sympathetic nerve were separated from the vagus nerve and prepared for stimulation. In some animals the separation of the two nerves was continued above the superior cervical ganglion so that postganglionic stimulation was possible. In a few cats the right sympathetic nerve was also isolated. The contraction of the nictitating membrane in response to nerve stimulation was recorded on smoked paper with an isotonic frontal-writing lever.

When changes in splenic volume were to be recorded, the abdomen was opened in the midline and the spleen was exposed. The vessels running between the spleen and the stomach were cut between ligatures. In this way the spleen was mobilized and, after the splenic nerves had been isolated, it was enclosed in an oncometer. Changes of volume were recorded with a piston volume recorder.

The amount of noradrenaline liberated in response to splenic nerve stimulation was determined by the technique of Brown and Gillespie (1957). The abdomen was opened and the spleen mobilized as described above. The inferior and superior mesenteric arteries were ligated and the intestines were removed from the lower part of the colon to the upper part of the duodenum. A polyethylene cannula, treated with silicone to inhibit clotting, was inserted into the superior mesenteric vein pointing towards the liver, and a loop of thread passed round the portal vein a few millimetres nearer to the liver than to the junction of the splenic and mesenteric veins. The splenic nerve was isolated, divided near the coeliac ganglion and prepared for stimulation. In most experiments the coeliac ganglion was excised, and where this was not done the splanchnic and hepatic nerves were divided. The adrenal glands were removed in four cats, but this procedure did not materially affect the concentration of noradrenaline in the plasma. Apart from the samples collected, blood loss during the experiments was slight, but in some instances the arterial pressure fell appreciably during the operation. In these cats 15 to 25 ml. of warm saline was slowly injected intravenously for 30 to 40 min. so that the experiments could be continued under reasonably stable conditions.

Plasma Samples.—As stimulation of the splenic nerve was begun tension was put on the loop around the portal vein and blood was collected for the 10 sec. of stimulation and for the following 20 sec. Sufficient heparin was included in each tube to prevent clotting. Blood in excess of 6 ml. was returned to the animal to reduce exsanguination. The retained blood was cooled and the plasma separated. The plasma was refrigerated until the following day, when its noradrenaline content was estimated using the blood pressure of a pithed rat which had received atropine. The haematocrit value of each sample of blood was measured in order that the output of noradrenaline/min. might be calculated.

Nerve Stimulation.—The cervical sympathetic nerve was laid across the electrodes in a pool of liquid paraffin. It was stimulated for 1 sec. in every minute by a Palmer Student Stimulator at a rate of 30 pulses/sec. of 4 V. and duration of 0.35 msec. In experiments in which samples of blood were collected, the splenic nerve was given similar stimuli, but the timing was 10 sec. in every 10 min. When changes in the splenic volume were being recorded the nerve was stimulated for periods of 2 sec. and the stimulus strength was adjusted to give responses commensurate with the capacity of the recording apparatus.

Solutions.—All drugs were dissolved in isotonic saline. Doses were given into a cannula in the femoral vein.

Results

Experiments on the Nictitating Membrane of the Cat

Actions of Choline Xylyl Ether.—In six experiments choline xylyl ether (10 mg./kg. body weight) produced a biphasic effect on the contractions of the nictitating membrane in response to nerve stimulation. *Phase one* was an immediate reduction in the size of the contraction lasting for about 8 min. In some experiments, the contractions entirely disappeared during the first 2 to 3 min. after injection and then reappeared and increased until they were nearly the same height as they were before the drug was given. In other experiments, the contractions were reduced to about one-third of their initial height. *Phase one* was reduced or absent when the amount of choline xylyl ether used was decreased to 5 mg./kg. *Phase two* consisted of a gradual diminution in the size of the contractions of the nictitating membrane until after varying intervals they disappeared altogether (Fig. 1). This phase usually began after 8 min. or more, but in one of the cats with the larger dose of choline xylyl ether it appeared to follow *phase one* immediately, and no contraction was recorded 2 min. after injection of the drug. When preganglionic stimulation had failed to elicit a response, stimulation of the postganglionic fibres was without effect. With doses of 10 mg./kg. of choline xylyl ether the average time for complete disappearance of contractions was 16 min. (range, 2 to 30 min., n=5). With doses of 5 mg./kg. *phase two* was prolonged and the average time for disappearance of contractions was 69 min. (range, 20 to 107 min.).

It was considered that frequent stimulation of the nerve might hasten the disappearance of contractions, and in two cats this hypothesis was tested. Both cervical sympathetic nerves were prepared for stimulation, and the right nerve was stimulated for 1 sec. in every minute for 1 hr. Stimulation of the left nerve was begun and 10 mg./kg. choline xylyl ether injected immediately afterwards. In both cats there was a slightly faster fall in the height of the contractions on the right side, but the difference between the two sides was small. Choline xylyl ether therefore appeared to exert the same effect whether or not the nerve was stimulated before the drug was given.

The Effects of Atropine on Sympathetic Nerve Block Produced by Choline Xylyl Ether.—In early experiments all animals received atropine (2 mg./kg., intravenously) to prevent any response of the nictitating membrane to stimulation of cholinergic fibres (Bacq and Fredericq, 1935), and to prevent any muscarine actions of choline xylyl ether itself. This rather large dose of atropine caused a diminution of the size of contraction of the nictitating membrane in eleven out of twelve cats.

Experiments were performed to determine whether or not atropine influenced the time taken by choline xylyl ether to prevent completely the

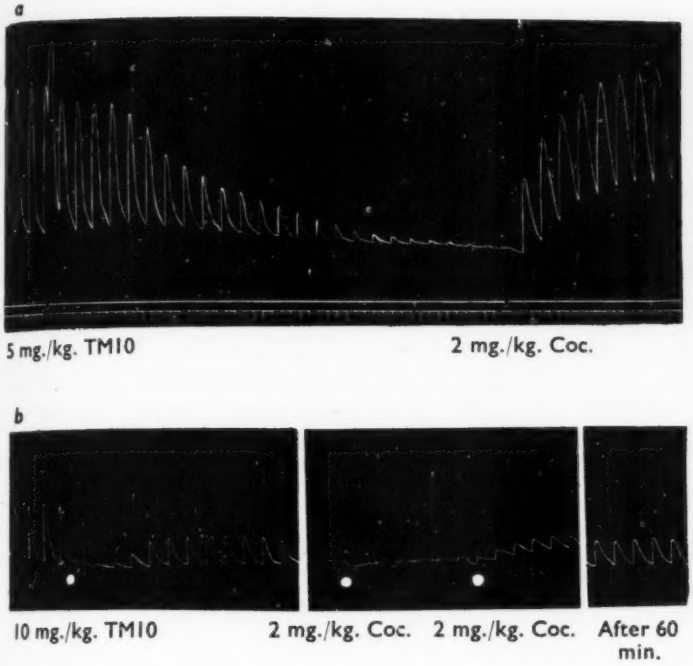

FIG. 1.—The effect of choline xylyl ether (**TM10**) on the responses of the nictitating membrane to supramaximal stimulation of the cervical sympathetic nerves in a cat anaesthetized with chloralose. ***a***, Shows reversal of the block produced by 5 mg./kg. of choline xylyl ether by 2 mg./kg. of cocaine. ***b***, Shows cocaine to be less effective in reversing the action of 10 mg./kg. of choline xylyl ether. The first injection of cocaine in ***b*** was given 30 min. after choline xylyl ether; the last section of the tracing was obtained 60 min. after injecting cocaine. Time, 30 sec.

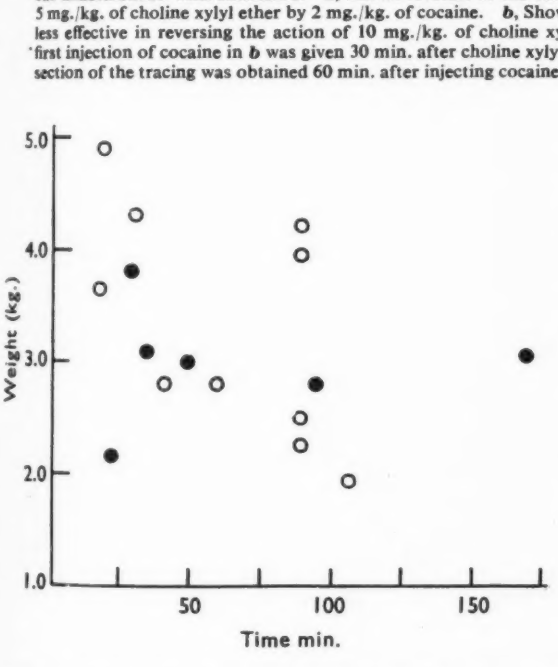

FIG. 2.—The variation in the time taken by a dose of 5 mg./kg. of choline xylyl ether to eliminate the responses of the nictitating membrane to stimulation of the cervical sympathetic nerve. ●, in the presence of 2 mg./kg. of atropine. O, in the absence of atropine.

response of the nictitating membrane to nerve stimulation. It also seemed possible that the time course of the effect of choline xylyl ether was not necessarily directly related to the dose, calculated on a body weight basis. In these experiments, the time taken by 5 mg./kg. of choline xylyl ether to block the responses of the nictitating membrane to stimulation of the cervical sympathetic nerve was compared between cats given 2 mg./kg. of atropine and cats not given atropine. The results of these experiments are plotted in Fig. 2 so that they indicate both the influence of atropine and of the dose (on a body weight basis) on the action of choline xylyl ether. Although the results could be interpreted to mean that atropine accelerated the action of the choline xylyl ether, any such effect was small and the routine administration of atropine was discontinued.

The Action of Cocaine.—There was antagonism between the effects of cocaine and choline xylyl ether on *phase two* but not on *phase one* of the action of the latter drug on the nictitating membrane.

When 2 mg./kg. of cocaine was given intravenously there was an immediate increase in the size of the contraction of the nictitating membrane followed by a rise in the base line due to prolongation of the effects of stimulating the cervical sympathetic nerve. In one experiment in which 5 mg./kg. of choline xylyl ether was given after cocaine, there was no change in the size of the contraction over the ensuing 100 min. In three experiments, 10 mg./kg. of choline xylyl ether was injected after cocaine. The size of the contraction was immediately reduced. This corresponded to *phase one* of the effects of large doses of choline xylyl ether already referred to, and no difference was detected between this *phase one* reaction and that occurring in the absence of cocaine. In two of the experiments after recovery from *phase one* due to choline xylyl ether, there was no reduction in the height of the contractions. In one experiment the recovery from *phase one* was not complete, and when further doses of choline xylyl ether were given the contractions were diminished and it was inferred that antagonism between the drug and cocaine was not absolute.

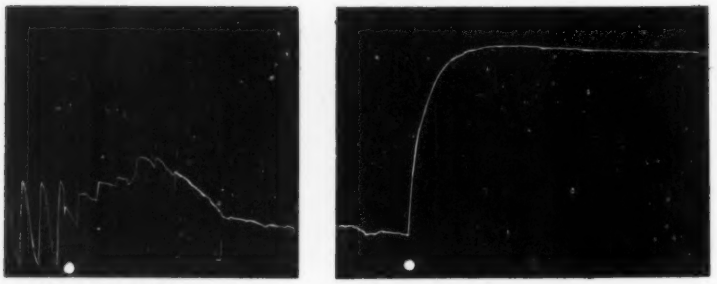

FIG. 3.—Contraction of the nictitating membrane in response to supramaximal stimuli applied to the cervical sympathetic nerve for 1 sec. every minute. Choline xylyl ether blocked the responses in 17 min.; the injection of cocaine then produced a contracture which was maintained only so long as stimulation at regular intervals was continued.

After the membrane response had been abolished by 5 mg./kg. of choline xylyl ether, the administration of cocaine led to an immediate return of the contraction to its former height, or almost so, within 10 min. After larger amounts of choline xylyl ether, cocaine caused recovery to only a third or a quarter of the former height of contraction. Further injections of cocaine tended to increase the height of the contraction, but in no experiment did it increase to more than half of the original height. These effects are illustrated in Fig. 1.

The antagonism between cocaine and choline xylyl ether appeared to be dependent upon the quantities of the two drugs present. Three cats were given 5 mg./kg. of choline xylyl ether and when the membrane response had disappeared 2 mg./kg. of cocaine was given. In each case the response was restored, but a further dose of 10 mg./kg. of choline xylyl ether abolished the response in two cats in 113 and 78 min. respectively, whilst in the third animal the response was only slightly diminished after 131 min. The third cat appeared to be less sensitive to choline xylyl ether since the times taken for inhibition of the membrane response after the first doses of choline xylyl ether were 23, 45, and 100 min. in the three cats respectively. It was also noted in two other experiments in which 10 mg./kg. of choline xylyl ether had been given previously, that although it had been impossible to restore the membrane response to its original value by a single injection of cocaine, three successive injections of 2 mg./kg. of cocaine each progressively increased the strength of the contractions.

In three of the fifteen experiments in which cocaine was injected after choline xylyl ether it produced a contracture of the nictitating membrane and not a restoration of the normal responses. This is illustrated in Fig. 3. The contracture was dependent upon regular nerve stimulation, for it slowly declined when stimulation was discontinued, and again became apparent when stimulation was restarted.

Experiments on the Spleen

The Effect of Choline Xylyl Ether on the Splenic Response to Nerve Stimulation.—Stimulation of the splenic nerve produced a decrease in splenic volume. In three experiments the injection of 5 mg./kg. of choline xylyl ether reduced the response to nerve stimulation after 10 min. and after 20 min. the response was practically abolished. The effects of a dose of 10 mg./kg. of the drug were similar and are illustrated in Fig. 4. This inhibition in about 20 min. occurred whether or not the stimulation was regular during that time. This confirmed the observations on the nictitating membrane that regular stimulation of the nerve fibre before the

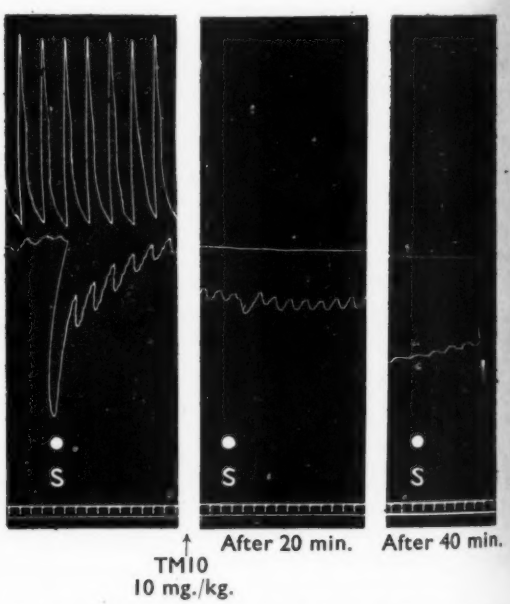

FIG. 4.—Upper record: contractions of the nictitating membrane in response to supramaximal stimulation of the cervical sympathetic nerve (for 1 sec. in every 60 sec.). Lower record: contractions of the spleen in response to stimulation of the splenic nerves (S) for 2 sec. in every 10 min. The injection of 10 mg./kg. of choline xylyl ether (T.M10) completely abolished the responses of the nictitating membrane in 20 min. At this time the splenic response was slight and it disappeared completely after 40 min. Time, 30 sec.

injection of choline xylyl ether did not hasten the effects of the drug.

The injection of 10 mg./kg. of choline xylyl ether after the animal had been given 2 mg./kg. of cocaine produced only a small reduction in the response of the spleen to stimulation of the splenic nerves 43 min. later. This is illustrated in Fig. 5. The animal from which this record was taken was not atropinized and the injection of choline xylyl ether produced a transient muscarine effect.

The Effect of Choline Xylyl Ether on the Secretion of Noradrenaline at Splenic Nerve Endings.—The experiments were divided into two groups. In both groups atropine was given at the beginning of the experiment and samples of blood were taken both in the resting state and during nerve stimulation. In the first group of experiments cocaine was injected before choline xylyl ether and in the second group the order was reversed. The results are given in Table I. The resting and stimulation samples for the control periods of both groups of experiments have been combined. It is apparent that when choline xylyl ether was injected before cocaine it greatly reduced the secretion of noradrenaline, and the amount of noradrenaline recovered from the blood was the same in the second stimulation sample taken 30 min. after the administration of choline xylyl ether as in the resting sample taken only 10 min. after the drug. Cocaine, given before choline xylyl ether, raised the amount of noradrenaline liberated by nerve stimulation. However, there were insufficient experiments to be certain that this increase in noradrenaline was significant. The injection of cocaine before choline xylyl ether prevented the latter drug from reducing noradrenaline liberation to the low levels recorded in its absence, but there was some reduction of the secretion. When cocaine was administered after choline xylyl ether had blocked the effects of nerve stimulation there was some recovery of the secretion of noradrenaline. The antagonism between choline xylyl ether and cocaine seen on the nictitating membrane response was therefore also seen on the spleen.

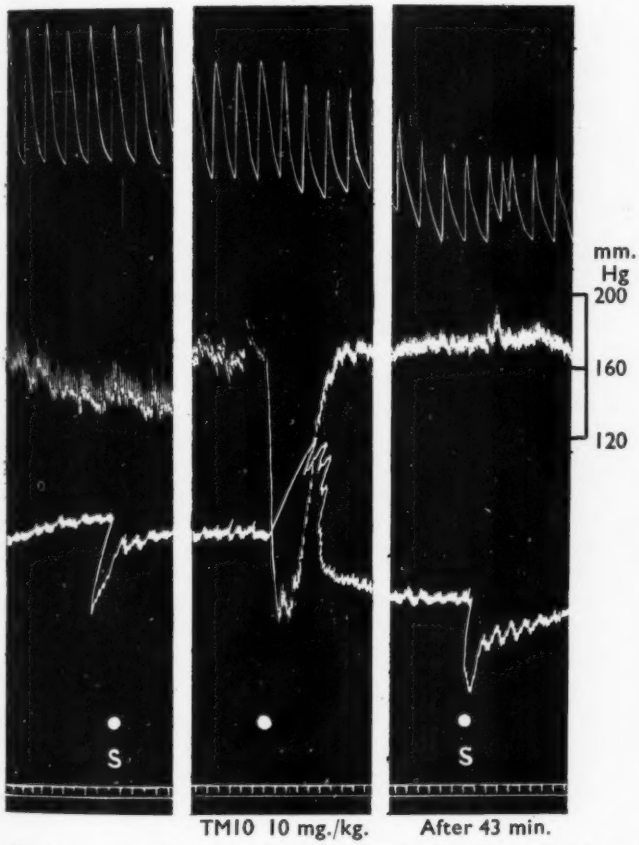

FIG. 5.—Cat given 2 mg./kg. of cocaine intravenously. Upper record: contractions of the nictitating membrane in response to supramaximal stimulation of the cervical sympathetic nerve for 1 sec. in every minute. Middle record: blood pressure. Lower record: contraction of the spleen in response to stimulation of the splenic nerves (S). The injection of choline xylyl ether (TM10) produced transient muscarine effects. The contractions of the nictitating membrane and the spleen were not blocked 43 min. after the injection of choline xylyl ether. Time, 30 sec.

The estimates for the noradrenaline in the resting samples in Table I are high. A few experiments were performed on animals from which the adrenal glands had been removed in an attempt to determine whether or not the glands were the source of this high background. There was no significant reduction of the resting noradrenaline in these experiments, and it was concluded that the high background was a consequence of the repetitive stimulation of the cervical sympathetic nerve, which was a constant feature of these experiments.

In one experiment phenoxybenzamine was given to prevent the adsorption of noradrenaline on the receptors. The order in which drugs were given was: choline xylyl ether, 5 mg./kg.; phenoxybenzamine, 10 mg./kg.; and cocaine, 2 mg./kg. After the choline xylyl ether there was no increase in the noradrenaline secreted when the splenic nerves were stimulated. The injection of phenoxybenzamine did not restore secretion which was, however, restored when cocaine was injected. All blood samples were in duplicate.

TABLE I

THE INFLUENCE OF CHOLINE XYLYL ETHER AND COCAINE ON THE RELEASE OF NORADRENALINE AT SPLENIC NERVE ENDINGS

Splenic venous blood was collected for noradrenaline assay during resting periods and after stimulation of the splenic nerve. The numerals are estimates of the amount of noradrenaline liberated into splenic venous blood during a period of 1 min. There was a 10 min. interval between the injection of a drug and collection of the first (resting) sample, and also between the collection of stimulation samples.

	Noradrenaline Release (ng./min.)				
	Combined Controls	Choline Xylyl Ether Alone	Cocaine Alone	Choline Xylyl Ether after Cocaine	Cocaine after Choline Xylyl Ether
Resting	223	68	213	173	95
First stimulation ..	391	121	562	358	165
Second ,, ..	443	70	528	360	147
No. of cats ..	9	5	4	4	5

DISCUSSION

The observation of Hey and Willey (1954) that choline xylyl ether blocks the response of the nictitating membrane to stimulation of the cervical sympathetic nerve has been confirmed. It has also been shown that with doses of 10 mg./kg. of choline xylyl ether the effect on the responses of the nictitating membrane was biphasic in the sense that there was an initial transient inhibition before the drug caused complete block. It seems probable that the transient inhibition of the membrane responses is due to two factors, both of which were demonstrated by Exley (1957). The first of these is a transient ganglion-blocking action; the second is a temporary depression of the responses of the membrane to adrenaline. Hey and Willey (1954) demonstrated also that choline xylyl ether temporarily inhibits the rise in blood pressure which normally follows an injection of adrenaline.

There was a great variation in the time taken in different animals for choline xylyl ether to block completely the nictitating membrane response. Bain and Fielden (1957) showed that the drug inhibits the synthesis of noradrenaline from dopamine, and it might be supposed, therefore, that the time taken by choline xylyl ether to block the effects of stimulating a nerve fibre would be proportional to the time taken to deplete the store of noradrenaline in the fibre. In our experiments repeated stimulation of the nerve for 1 sec. in every minute for 1 hr. before the injection of choline xylyl ether did not materially affect the time taken to produce block. Had regular stimulation of the nerve before the injection of the drug reduced the time taken to produce block, it would have indicated that the variations were due to differences in the amount of stored noradrenaline. It is possible that in our experiments the rate of synthesis or replacement of the noradrenaline in the nerve fibre was at least equal to the rate of its liberation.

The action of atropine in these experiments is more difficult to assess. In the absence of atropine an injection of cocaine prevented choline xylyl ether, given subsequently, from exerting its effect. In the presence of atropine, however, cocaine delayed but did not abolish the effect of choline xylyl ether. It is possible that atropine and cocaine, having somewhat similar molecules, competed for the same receptors.

Bacq and Fredericq (1935) have shown that, when physostigmine was instilled into the conjunctival sac of the eye of a cat, the responses of the nictitating membrane to stimulation of the cervical sympathetic nerve were increased. Furthermore, the responses were inhibited by intravenous doses of atropine, which latter observation we have confirmed in the present work. It was therefore suggested that there might be some cholinergic innervation of the nictitating membrane. However, since Exley (1957) showed that choline xylyl ether blocked the effect of sympathetic but not of vagal stimulation on the cat heart, the observation that this drug will completely block the nictitating membrane response to nerve stimulation in the absence of atropine casts some serious doubt upon the possibility of there being any cholinergic fibres present. The diminution in contraction of the nictitating membrane after atropine might be better correlated with the observation of Thompson (1958) that the responses of the isolated nictitating membrane to doses of adrenaline are reduced by atropine, which seems to imply that the action of atropine in this respect is directly on the muscle.

In confirmation of the results of Exley (1957) we have shown that choline xylyl ether prevents the secretion of noradrenaline at splenic nerve endings. The previous injection of cocaine antagonized this effect. After choline xylyl ether had prevented the secretion of sympathin, cocaine partially restored it. The antagonistic action of cocaine was reflected in the pattern of events observed when the cervical sympathetic nerve was stimulated and the contractions of the nictitating membrane were recorded.

The disappearance of noradrenaline from the plasma in the splenic vein after a dose of choline xylyl ether cannot be explained by postulating a bigger uptake of noradrenaline by the receptors: when the receptors were blocked with phenoxybenzamine (Brown and Gillespie, 1957; Brown, Davies, and Gillespie, 1958) the effect was merely

to increase the resting concentration of noradrenaline in the plasma and not to restore the response to nerve stimulation.

Brown and Hey (1956) found that various nuclear-substituted choline phenyl ethers, including choline xylyl ether, inhibited the enzymatic destruction of adrenaline by guinea-pig liver. It is therefore highly unlikely that choline xylyl ether works by promoting rapid enzymatic destruction of noradrenaline, though if this were so the action of cocaine would be easy to explain, as it has been shown to antagonize the action of amine oxidase *in vitro* (Philpot, 1940).

There is some evidence that choline xylyl ether interferes with the synthesis of noradrenaline. Coupland and Exley (1957) showed that the injection of choline xylyl ether into rats depleted the adrenal glands of their stores of adrenaline and noradrenaline. Furthermore, Bain and Fielden (1957) reported that choline xylyl ether inhibited the synthesis of noradrenaline from dopamine by homogenates of human chromaffin-cell tumours. Earlier Bain and Fielden (1956) had demonstrated that dopamine antagonized the blocking action of choline xylyl ether and that the latter did not inhibit the synthesis of dopamine itself. It has, therefore, to be explained why added dopamine did not antagonize the action of choline xylyl ether in the homogenate. One possible interpretation of the facts, so far as they are known at present, is that choline xylyl ether can enter into combination with dopamine and with cocaine. The effect of the combination with dopamine would be to prevent the formation of noradrenaline unless an excess of dopamine is provided. Combination of choline xylyl ether with cocaine would effectively prevent the former from combining with dopamine.

Our thanks are due to Messrs. May & Baker Ltd. for generous supplies of choline 2,6-xylyl ether bromide and to Messrs. Smith, Kline & French for the Dibenzyline (phenoxybenzamine). We are grateful to Dr. H. C. Stewart for his interest in the work and to Miss E. Morris and Miss M. Trent for technical assistance.

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ANTIPHLOGISTIC ACTIVITY OF IPRONIAZID

BY

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Iproniazid was found to inhibit formalin-induced oedema of the foot, dextran-induced oedema and cotton-pellet-induced granulomatous tissue in the rat. The presence of the adrenals was essential for the antiphlogistic activity. The antiphlogistic action was not accompanied by any antipyretic effect. Inhibition of formalin-induced oedema was also observed with phenylbutazone and with salicylamide.

Little is known about the therapeutic efficacy and mechanism of action of iproniazid in rheumatoid arthritis. In the opinion of Scherbel (1957, 1958a and b), the main effect of the drug is to improve the psychic condition of patients. However, Banghart (1958), using higher doses, frequently obtained objective improvement, but he also observed side-effects in a large number of patients and frequently had to reduce the dose or even suspend treatment with the drug.

This paper describes a pharmacological investigation of the mechanism of action of iproniazid in artificially induced inflammatory states.

METHODS

Antiphlogistic Action

Formaldehyde-induced Oedema. — Oedema was induced in the hind foot by the injection of 0.1 ml. of a 3% solution of formaldehyde into the plantar aponeurosis of male rats (150 to 250 g.). The increase in the dorsoplantar thickness of the injected foot was determined at hourly intervals for 6 hr. by a calliper rule applied at a level between the two proximal and the two medial plantar eminences. With a little experience the calliper jaws could be applied at a constant pressure and the measurements of thickness were reproducible within ± 0.1 mm. Drugs were injected subcutaneously immediately before the injection of formaldehyde.

Dextran-induced Oedema.—Oedema was induced by the intraperitoneal injection of 300 mg./kg. of dextran into male rats (150 to 250 g.). Oedema in the hind feet was measured as described above. Iproniazid was injected subcutaneously 30 min. before the administration of dextran.

Cotton-pellet-induced Granulomata.—The weights of the granulomata, which formed after the subcutaneous insertion of cotton pellets weighing 18 to 19 mg. into male rats weighing 70 to 80 g., were estimated by the method of Meier, Schuler, and Desaulles (1950). Administration of iproniazid was started the day after implantation, and either 50 or 100 mg./kg. of the drug was given subcutaneously daily for six days. On the eighth day after implantation the rats were killed, the granulomata removed, freed from the cotton nucleus and wet-weighed.

Antipyretic Action

This was tested by the subcutaneous injection of the drugs into rabbits which received immediately afterwards an intravenous injection of 1 ml. of anti-typhoid vaccine mixed with 1 ml. of a 20% suspension of brewers' yeast. Rectal temperatures were measured by thermocouples.

RESULTS

Formaldehyde-induced Oedema. — Iproniazid in doses of 200 mg./kg. almost completely inhibited a formaldehyde-induced oedema (Fig. 1). With doses of 100 and 50 mg./kg., the inhibition was less marked but statistically significant. The intensity of the antiphlogistic action was expressed quantitatively by measuring the area enclosed between the curves for the control and treated animals (Figs. 1 and 2). The logarithm of the dose and the surface (expressed in arbitrary units) of these areas are clearly proportional (Fig. 3).

The antiphlogistic effects of phenylbutazone and salicylamide are represented in Fig. 2. It was not possible to calculate a potency ratio between iproniazid and phenylbutazone because the slopes of the dose/activity lines are different. However, iproniazid seemed to be somewhat more active than phenylbutazone and far more active than salicylamide with this test. Moreover, since the intraperitoneal LD50 of iproniazid was 382 mg./kg. (range 456 to 320), while that of phenylbutazone was 266 mg./kg. (284 to 248), using the

method of Litchfield and Wilcoxon (1949), the therapeutic index of iproniazid seems to be decidedly better than that of phenylbutazone.

Dextran-induced Oedema.—The inhibition of dextran-induced oedema by iproniazid seemed to be less intense and more fleeting than that of oedema induced by formaldehyde (Fig. 4). Indeed, at the end of the second hour there was no statistically significant difference between the control and treated rats.

Cotton-pellet-induced Granulomata.—The administration of both 50 and 100 mg. of iproniazid/kg. for six days led to a decrease in the weight of foreign body granulomata which was both statistically significant and proportional to the dose (Table I). Control measurements of body weight and of the weight of various internal organs showed a decrease in the weight of the thymus, though this was only statistically significant after the daily dose of 100 mg./kg.

Adrenal Function.—To check the effects of iproniazid upon adrenal function 50 mg./kg. of the drug in three equal doses was given subcutaneously to rats of both sexes with body weights of 140 to 160 g. After 24 hr., no change in the weight of the adrenals could be detected nor any alteration in their ascorbic acid and cholesterol contents as compared with the controls. This applied both to starved rats and to rats subjected to immobilization stress (Setnikar and Murmann, 1958). The thymus weight always

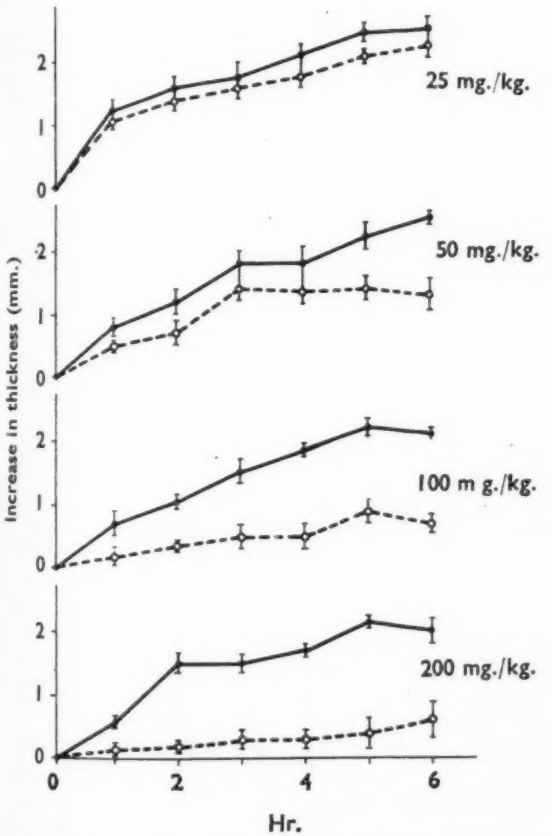

FIG. 1.—Inhibition of the increase in thickness of the hind feet of rats caused by the injection of formaldehyde with graded subcutaneous doses of iproniazid. Every dose was tested on ten rats and there were ten controls. Statistically significant differences between treated rats and controls are observed with doses of iproniazid of 50 mg./kg. and over. Solid lines, controls; broken lines, treated rats.

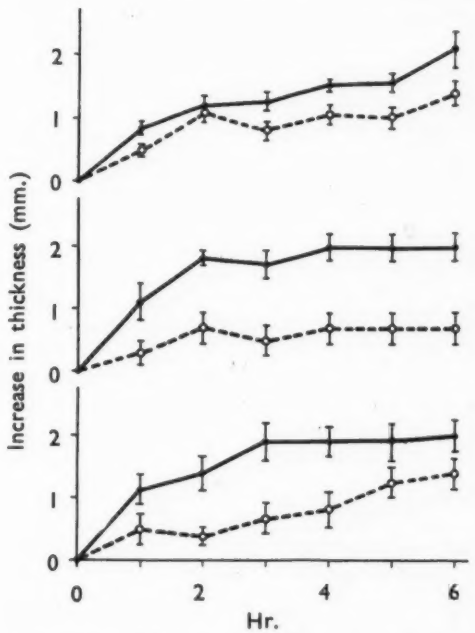

FIG. 2.—Inhibition of formalin-induced plantar oedema by phenylbutazone and salicylamide given subcutaneously. Solid lines, controls; broken lines, treated rats. Upper curves, phenylbutazone, 135 mg./kg.; middle curves, phenylbutazone, 200 mg./kg.; lowest curves, salicylamide, 500 mg./kg.

TABLE I

INHIBITION OF GRANULOMA FORMATION BY IPRONIAZID

Daily Dose of Iproniazid (mg./kg.)	No. of Rats	No. of Granulomata	Average Weight of Granulomata (mg.)	Difference between Controls and Treated Rats	Probability of Difference (P)
0	10	40	167±2		
50	10	40	153±3	−8%	<0·001
0	10	40	162±5		
100	10	40	143±2	−12%	<0·001

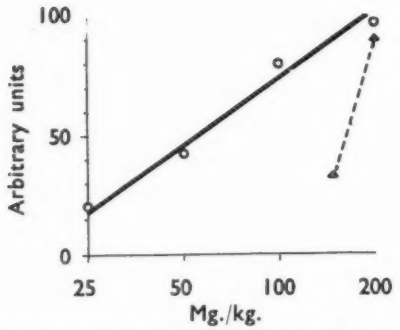

FIG. 3.—Antiphlogistic effect of iproniazid (solid line) and phenylbutazone (broken line). Ordinate: arbitrary units measuring the areas enclosed by the curves for treated animals and controls in Figs. 1 and 2. Abscissa: logarithm of the dose. Note that the slopes of the activity/log dose lines are very different for the two drugs.

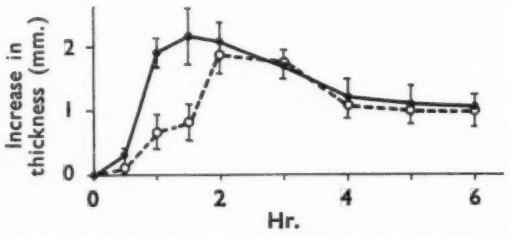

FIG. 4.—Effect of 100 mg./kg. of iproniazid (broken line) on the increase in dorso-plantar thickness of the hind feet of rats to which 300 mg./kg. of dextran had been given intraperitoneally. Maximum oedema occurred 90 min. after injection. The maximum difference between controls (solid line) and animals treated with iproniazid can be observed during the same period. Each point is the average value from 14 rats.

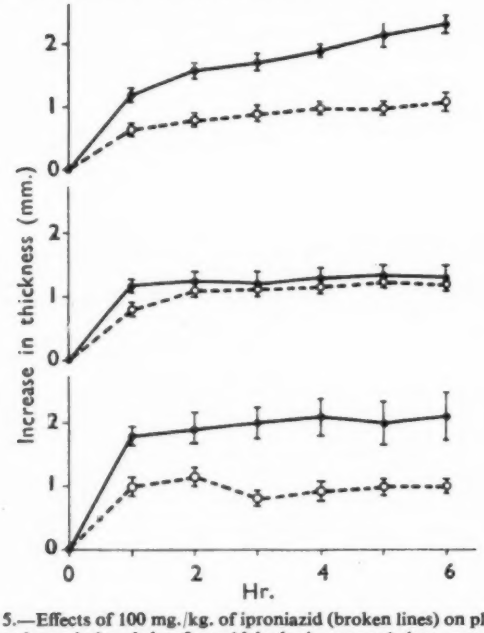

FIG. 5.—Effects of 100 mg./kg. of iproniazid (broken lines) on plantar oedema induced by formaldehyde in normal (upper curves), adrenalectomized (middle curves), and hypophysectomized (lower curves) rats.

showed a decrease, but this was not statistically significant.

Formaldehyde-induced Oedema in Adrenalectomized and Hypophysectomized Rats.—Iproniazid (100 mg./kg. subcutaneously) exerted a clear antiphlogistic effect in hypophysectomized rats, comparable to that in normal animals, but after adrenalectomy the drug had practically no action; only after the first hour was there a small, but statistically significant, difference ($0.02<P<0.05$) between treated rats and controls (Fig. 5). It seems therefore that the adrenals are necessary for the antiphlogistic activity of iproniazid.

Antipyretic Action.—Iproniazid up to 200 mg./kg. subcutaneously exerted no antipyretic action in hyperthermal rabbits, whereas the action of phenylbutazone was evident with 100 mg./kg. doses.

DISCUSSION

The antiphlogistic action of iproniazid in doses of 50 mg./kg. or more may explain the antirheumatic activity which, according to Scherbel (1957, 1958a and b) and Banghart (1958), follows large and toxic doses of this drug.

Analogous antiphlogistic effects are observed with similar substances such as isoniazid (Theobald, 1955). For isoniazid too it has been noted that the antiphlogistic action is effective in hypophysectomized animals and is much less in adrenalectomized animals (Domenjoz, 1954).

The hypothesis might be advanced that iproniazid acts by inhibiting the degradation of 5-hydroxytryptamine, which also has an antiphlogistic effect (Georges and Herold, 1957). On the other hand, while both iproniazid and 5-hydroxytryptamine act only in the presence of the adrenals, the former does not seem to affect either normal or stress-stimulated adrenal activity, whereas the latter has an antagonistic effect upon the reduction of ascorbic acid caused by stress (Georges and Herold, 1958; Georges, 1957). It is therefore possible that the antiphlogistic action of 5-hydroxytryptamine is due to a modification of the hormonal secretion of the adrenals while that of iproniazid may be due to another mechanism, for example, an inhibition of hepatic degradation of adrenal antiphlogistic hormones, a mode of action which has already been invoked to explain the antiphlogistic action of isoniazid (Wiesel, 1956; Wiesel, Barritt and Scheid, 1956).

In conclusion, some of the antirheumatic effects of large doses of iproniazid in man seen by Scherbel (1957, 1958a and b) and by

Banghart (1958) may be explained by the antiphlogistic action. It is therefore probable that, in rheumatoid arthritis, iproniazid exerts a direct therapeutic action on the lesions and not merely an indirect action, namely an improvement in psychic condition as suggested by Scherbel (1958a and b). But it must be admitted that this direct therapeutic effect can be obtained only with very high toxic doses of the drug which limits its therapeutic usefulness.

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EXPERIMENTAL HEPATIC AMOEBIASIS AND ITS APPLICATION TO CHEMOTHERAPEUTIC STUDIES

BY

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Induction of hepatic amoebiasis with two strains of *Entamoeba histolytica* has been attempted by direct inoculation of trophozoites into the livers of rats, hamsters, and guinea-pigs, and by intravenous injection into guinea-pigs. A high incidence of amoebic abscesses was obtained in hamsters. Rats and guinea-pigs were relatively less susceptible. The two strains of *Entamoeba histolytica* differed in virulence. Serial liver passage increased their virulence for the hamster liver, and also increased invasiveness to the rat caecum. The chemotherapeutic effects of chloroquine, emetine, and some anilides were determined. The procedure finally adopted was based on mean survival times of treated and untreated animals. Chloroquine and emetine were effective under conditions which greatly favoured the drugs, but the anilides showed little effect.

Early attempts in the laboratory to induce hepatic amoebiasis were successful, using dogs and cats (Cleveland and Sanders, 1930a; Kondo, 1939), but rats and guinea-pigs are relatively insusceptible (Rao, 1951; Yoshimura, 1952; Maegraith and Harinasuta, 1953, 1954a, 1954b), though hamsters gave more encouraging results (Reinertson and Thompson, 1951; Neal and Vincent, 1955, 1956). Successful treatment of hepatic amoebiasis in hamsters was described by Thompson and Reinertson (1951) and by Neal and Vincent (1955).

In this laboratory it was hoped to use hepatic amoebiasis for further chemotherapeutic studies on a series of compounds found active *in vitro*, and in experimental intestinal amoebiasis in rats (Bristow, Oxley, Williams, and Woolfe, 1956). As we normally used rats and guinea-pigs for studies of intestinal amoebiasis, initial attempts were made to establish hepatic abscesses in these species. Later, hamsters were employed with much greater success.

METHODS

Rats, hamsters, and guinea-pigs of both sexes were used. The rats were approximately four weeks old and weighed about 60 g. The hamsters were five to six weeks old and weighed 45 to 55 g. The guinea-pigs were adult.

Two strains of *Entamoeba histolytica* of human origin were used. These were strain M (obtained originally from Dr. R. A. Neal of the Wellcome Foundation), and strain R (obtained from the Liverpool School of Tropical Medicine). Strain M had been in culture for some years; strain R was of more recent origin. All strains and substrains were maintained in culture with their respective bacterial flora at 37°. The cultures were subcultured every other day, alternately on a diphasic medium (egg-slope with a serum-saline (1:6) overlay at *p*H 7.3) and on a monophasic medium (liver-Marmite-serum) at *p*H 7.4. Rice starch was added to each tube.

To infect the animals, the technique of Reinertson and Thompson (1951) was followed. Initially 20,000 to 50,000 *E. histolytica* trophozoites with the concomitant bacteria from a 48 hr. culture were inoculated in a volume of 0.05 ml. Preliminary tests revealed a difference in infectivity of the two strains to rats, and thereafter strain R, the more infective of the two, was usually used. The inoculum was standardized at 8,000 trophozoites in 0.02 ml. and was thickened with rice starch to control "run back."

In some experiments prophylactic inoculations of bacteria were given to raise the resistance of the hamsters, using the method of Reinertson and Thompson (1951), before the amoebae and bacteria were injected. The inoculum, however, usually contained very few bacteria. It was obtained by culturing the amoebae plus bacteria with 0.02 mg./ml. of oxytetracycline in the monophasic medium for 24 hr. under N_2 in a McIntosh and Fildes jar. After counting the amoebae and standardizing the inoculum, sterile starch was added to thicken the medium. This method reduced very considerably the number of bacteria in the culture. Further bacterial control was carried out by subcutaneous injection of antibiotics (penicillin, 2 mg./kg., and streptomycin, 50 mg./kg.) into the inoculated animals. However,

this never produced bacteriological sterility of the amoebic abscesses.

For the experiments on the chemotherapy of experimental hepatic amoebiasis, hamsters infected with strain R of *E. histolytica* were used throughout. After a few preliminary experiments using 8,000 to 25,000 amoebae in the inoculum, the dose was standardized at 8,000 amoebae.

The hamsters were divided into groups for treatment and then selected at random (Thompson and Reinertson, 1951). In early experiments they were killed after four to six days and their livers examined for the presence of active amoebae in wet smears in sections. The effects of therapy were determined by comparison of treated with control animals.

Emetine hydrochloride was given intraperitoneally, and chloroquine sulphate and fumagillin were given orally. Emetine was administered once a day, and the other drugs twice daily, over a period of four days. An initial dose was given 6 hr. before inoculation, a second at the time of inoculation, and the remainder in the morning and afternoon on the following three days. This regimen proved inadequate and was finally altered to three doses before, and three to five after, inoculation. All hamsters were examined for the presence of active trophozoites in the liver abscesses as soon as practicable after death. Often, active amoebae were recovered from abscesses many hours after death of the animals. The lesions could not be cut out and weighed as described by Thompson and Reinertson (1951). We thus could not readily detect compounds which might partially suppress the abscesses, and mean survival time was therefore adopted as an alternative method of evaluation.

New drugs tested were nearly all anilides, analogues of diloxanide (Entamide, dichloroacet-*p*-hydroxy-*N*-methylanilide). All compounds were given in aqueous solutions or suspensions, by oral intubation under ether anaesthesia.

Results

Inoculation with Strain M

The incidence of lesions and recovery of *E. histolytica* in rats, hamsters, and guinea-pigs is given in Table I. Forty-eight out of eighty-four inoculated rats showed lesions when killed and examined four to fifteen days after inoculation. The lesions were from 0.1 cm. to 1.0 cm. in diameter and all had a definite wall. The small lesions were thick walled with creamy pus at the centre. Those intermediate in size also had thick walls but had a thick greenish paste in the centre. Eight of the lesions were large with a thin wall, and were full of a brown pus. Bacteria were present in all abscesses, but active amoebae were recovered only from the large thin walled abscesses, and only in those examined between four and six days after inoculation. Inoculation of the bacterial flora alone gave abscesses which were small, hard and thick walled and contained whitish pus.

TABLE I

INTRAHEPATIC INOCULATION WITH *E. HISTOLYTICA*

The inocula marked with an asterisk were given into the mesenteric or portal veins.

Strain	Species	No. of Amoebae Inoculated	Source of Trophozoites	Proportion with Lesions	Proportion in which *E. histolytica* were Found
M	Rats	20,000–50,000	Culture	48/84	8/84
	Hamsters	20,000–50,000	,,	22/33	14/33
		Approx. 6,000	Liver lesion	50/59	45/59
	Guinea-pigs	20,000–50,000	Culture	10/12	0/12
MLVe	Hamsters	20,000–30,000	,,	11/12 8/12	8/12 3/12
R	,,	10,000–40,000	,,	20/22	18/22
	Guinea-pigs	20,000–60,000	,,	16/16	0/16
		20,000–60,000*	,,	8/20	0/20
		Approx. 5,000	Liver lesions	8/14	0/14
RL1	Hamsters	20,000	Culture	12/12	12/12
RL2	,,	20,000	,,	12/12	12/12
RL5	,,	20,000	,,	12/12	12/12

In hamsters, the amoebic abscesses were quite different. The hamsters were killed and examined three to twenty-one days after inoculation. A few of the abscesses were of the walled-off type found in rats, but the majority were not encapsulated. Most of them were about 1 cm. in diameter, but often the whole of the inoculated lobe was affected with spread into other lobes. Active amoebae were recovered from individual abscesses from three to fifteen days after inoculation. These abscesses appeared as pale areas in the liver, many containing a reddish-brown paste or liquid full of bacteria and dead liver cells. While there was no wall at the periphery of the abscess, there was a sharp division between dead liver cells and healthy liver tissue. Amoebae were seldom found in the interior of the abscess, but they were usually found, in large numbers, at the junction between the abscess and healthy tissue. Twenty-two out of thirty-three inoculated hamsters had lesions, and active amoebae were recovered from fourteen of these.

These results showed that hamsters were more susceptible than rats, but the incidence of amoebic abscesses was too low to be used as a reliable test

for drug action. It has been shown that the virulence of strains of *E. histolytica* for rat and hamster liver and the rat caecum can be increased by passage through the rat intestine (Yoshimura, 1952), through the dog intestine (Thompson, McCarthy and Reinertson, 1954) and through the liver (Cleveland and Sanders, 1930b; Neal and Vincent, 1956). Instead of culturing the amoebae between liver passages, we attempted to raise the incidence of amoebic abscesses by repeated serial passage direct from liver to liver of hamsters before recovering the amoebae into culture for inoculation. Infected livers with active trophozoites were ground up in normal saline and 0.02 ml. of the suspension inoculated into the livers of fresh hamsters. The number of amoebae in the inoculum was considerably less than that used for the earlier experiments. Cleveland and Sanders (1930b) were apparently unable to accomplish direct liver passage and attributed their lack of success to too few trophozoites in the inoculum.

Table I shows that serial passage through the liver enhanced the infectivity of the amoebae, the incidence of amoebic abscesses being raised from 42% (14/33) to an overall incidence of 76% (45/59). After fourteen liver-to-liver passages the incidence rose to 95%. It proved difficult, however, to maintain in cultivation the amoebae recovered after the fourteenth passage, and they died after a few subcultures. A few hamsters were inoculated from cultures recovered from five to seven liver passages. The incidence of amoebic abscesses in these inoculations was 90%.

A small number of guinea-pigs were also inoculated with strain M, but no amoebic abscesses were obtained. Ten out of twelve guinea-pigs inoculated showed lesions, but no amoebae were recovered from these. The lesions were usually very small and hard and contained a little whitish pus. They were normally walled off from the rest of the liver, and appeared to be typical bacterial abscesses.

Some work was also done with strain MLVe (a liver-passaged substrain of strain M provided by Dr. R. A. Neal) because strain M had become very attenuated and was nearly non-invasive in the rat intestine. Strain MLVe infected the hamster liver better than did the original strain M, but the incidence of amoebic abscesses was still low (Table I), and fell fairly rapidly in the course of eight weeks.

Inoculation with Strain R

In the early stages of the experiments with strain M, when the incidence of amoebic abscesses was low, we obtained strain R which had been used by Maegraith and Harinasuta (1954a and b) in their work on guinea-pigs. This strain immediately proved highly virulent for the hamster liver. The type of lesion was almost invariably rapidly spreading without a wall. The abscesses were pale with a definite periphery, the interior consisting of a thin paste or liquid containing dead liver cells and bacteria—apparently the "anchovy paste" in human hepatic amoebiasis. Amoebae were found in large numbers round the periphery of the abscess, where there was an abrupt transition to healthy liver tissue. The infection killed the hamsters within six to ten days.

In guinea-pigs, though lesions were obtained both after intrahepatic inoculation and after injection into the mesenteric or portal vein, no amoebic abscesses were found. The abscesses were small and hard and appeared to be only bacterial, since no amoebae were recovered from any of these lesions, in contradistinction to the results of Maegraith and Harinasuta (1953, 1954a).

Direct liver-to-liver passage in hamsters was also carried out with this strain, and after one passage (RL1) the incidence of lesions was 100%. Strangely enough, the amoebae of the original strain R were not very invasive in the rat intestine. After five liver passages the amoebae were recovered into cultivation *in vitro* and were then very invasive in the rat. This substrain, RL5, has been in cultivation *in vitro* without liver passage for over a year. While it is still invasive for the rat intestine, its virulence is slowly declining.

Chemotherapy of Hepatic Amoebiasis

Emetine and chloroquine were used as control drugs. Table II shows the results of initial experiments with emetine, chloroquine, and fumagillin. The treatment failed to suppress the abscesses. Inoculation with smaller numbers of *E. histolytica* gave unreliable infections.

TABLE II

TREATMENT OF HEPATIC AMOEBIASIS (STRAIN R) WITH EMETINE, CHLOROQUINE, AND FUMAGILLIN

One dose given before, and the remainder after, inoculation. All doses given twice daily, except emetine (once a day). All hamsters were killed and examined five to six days after inoculation. A positive result means that amoebae were found in the lesion.

Drug	Dose (mg./kg.)	No. of Doses	No. of Amoebae Inoculated	Treated: Proportion Positive	Untreated: Proportion Positive
Emetine	6	3	10,000	6/11	5/5
	4	3	8,000	7/10	10/10
Chloroquine sulphate	100	6	8,000	9/11	10/10
			6,000	6/8	6/6
			3,000	2/8	4/6
Fumagillin	5	6	8,000	10/10	10/10
	2·5	6	8,000	10/10	10/10
	1·25	6	8,000	10/10	10/10

Table III shows the results of treatment with diloxanide and some of its esters. Several other analogues of diloxanide were also tested in the same way without any effect.

TABLE III

TREATMENT WITH DILOXANIDE AND SOME OF ITS ESTERS

Three doses before and three to five doses after inoculation, two doses given daily. All hamsters killed and examined six days after inoculation. Chloroquine controls were given 100 mg./kg. for the same number of doses as the drug on test. A positive result means that amoebae were found in the lesion. The drug marked with an asterisk is 7-chloro-4-(4-diethylamino-1-methylbutylamino)quinolinium di(4-dichloroacet-*N*-methyl amido)phenyl phosphate.

Drug	Dose (mg./kg.)	No. of Doses	No. of Amoebae Inoculated	Treated: Proportion Positive	Chloroquine Controls: Proportion Positive	Untreated: Proportion Positive
Diloxanide	100	6	5,000	4/6	2/6	4/6
	100	8	4,000	3/5	0/4	6/6
Diloxanide furoate	100	6	5,000	4/6	2/6	4/6
Diloxanide benzoate	100	6	5,000	4/6	2/6	4/6
Diloxanide stearate	100	6	5,000	3/6	1/6	6/6
RD5269*	100	6	5,000	3/5	2/5	5/5
	100	8	4,000	2/5	1/5	3/4

Table IV gives the results of tests with some compounds using mean survival times as the criterion of effectiveness. Emetine was found to be effective, while chloroquine was not consistently so. Fumagillin was inactive and somewhat toxic

TABLE IV

MEAN SURVIVAL TIMES AFTER DRUG TREATMENT

All doses given twice daily, three before, the remainder after inoculation, except emetine which was given once a day with two doses before and two after inoculation. All hamsters inoculated with about 8,000 amoebae. A positive result means that amoebae were found in the lesions. RD5269, see Table III.

Drug	Dose (mg./kg.)	No. of Doses	Proportion Positive	Mean Survival Time (Days)	
				Treated	Untreated
Emetine ..	6	5	0/10	7·5	10
	4	5	0/6	>33·0	10
	2	5	0/6	>33·0	10·25
Chloroquine	400	5	Toxic	0·75	8·0
	200	6	,,	1·25	8·0
	100	6	9/52	>22·0	7·5
	50	8	10/10	8·0	6·5
Fumagillin	25	6	6/8	4·5	10·25
	10	6	6/8	6·2	10·25
Diloxanide	200	8	6/7	7·1	6·4
	100	8	3/4	7·75	6·5
	25	8	3/4	7·4	6·5
RD5269 ..	100	6	4/5	12·6	9·0
Piperazine diloxanide sulphate	100	6	4/6	14·4	9·5
	100	8	2/5	>16	7·4
	25	8	6/7	8·3	7·4

at the doses used. Several analogues of diloxanide were tried, but none had any significant effect. One piperazine salt of a diloxanide ester was also tested. In addition a number of compounds, including some of those given in Tables III and IV, were tested using liver-passaged strains RL2, RL3, and RL5. These substrains were rather more virulent than the original strain R, and all animals were therefore examined five days after inoculation. The rapid spread of the infection made these substrains unsuitable for testing drugs.

DISCUSSION

Both rats and guinea-pigs are relatively insusceptible to hepatic amoebiasis. In our experiments with these species all the lesions in the liver after intrahepatic inoculation were encapsulated. Fibrosis round the margin of the liver abscess is reported in most of the experimental work published. This fibrosis occurs only rarely in amoebic abscesses in man (Craig, 1934). It may be that this wall of granulation tissue which limits the lesion is primarily a reaction against the bacteria, and only incidentally against the amoebae.

With hamsters, however, we found that a capsule or wall was seldom formed at the periphery of the abscess when amoebae were present and actively multiplying. When amoebae were found they were near the periphery of the abscess next to healthy liver tissue. When, rarely, the lesions were walled off, the appearance was that of a purely bacterial abscess, and if amoebae were present they were probably survivors of the original inoculum. This probably happens also in rats and guinea-pigs.

We found that the lesions set up in hamsters with strain R almost invariably had liquefied centres, and adhered to the peritoneum and diaphragm. This was found by Neal and Vincent (1956) after infection with one of their liver-passaged strains; they recovered from the lesions large numbers of amoebae which grew readily *in vitro*. On the other hand, Reinertson and Thompson (1951) found that the lesions caused in hamsters by their strain of *E. histolytica* were rarely purulent macro- or micro-scopically, although some bacteria were present, and the lesions were rarely liquefied. These authors consider that this indicated, *inter alia*, that the lesions were caused by the amoebae, and not by the associated bacteria. The lesions we obtained approximated more closely to those described in man.

There was a marked difference in virulence both in the same strain of *E. histolytica* towards

different species, and in the virulence of different strains towards the same host. The virulence of *E. histolytica* for both the rat liver and the rat caecum can be increased in a number of ways (Yoshimura, 1952; Thompson *et al.*, 1954; Cleveland and Sanders, 1930b; Neal and Vincent, 1956), the simplest probably being liver-to-liver passage. The infectivity of strain M towards the hamster liver was increased in this way, as was its invasiveness to the rat caecum. This recovery of invasiveness to the rat caecum after liver passage (Neal and Vincent, 1956) suggests that any strain of *E. histolytica* cultivated *in vitro* for use in a screening test in intestinal amoebiasis should be periodically passaged through the liver, so that it retains its ability to produce ulceration in the gut. Different strains, however, might require to be passaged at different intervals of time in order to maintain their invasiveness, as shown by the strain MLVe of Dr. R. A. Neal and our strain RL5. In the same way, consistently high incidence of infection towards the liver can be maintained by periodic liver passage of the strain cultivated *in vitro*.

The use of hamsters for the detection of agents effective in treating hepatic amoebiasis is certainly practicable, as shown by our results with emetine and chloroquine. The disadvantage, as pointed out by Thompson and Reinertson (1951), is that the test is a very severe one owing to the rapidity of spread of the lesions and early death of the animals. Reducing the number of amoebae inoculated delays the death of the hamsters, but this advantage is offset by less uniformity and dependability of the infections. In order to use mean survival times to demonstrate the pronounced effectiveness of emetine and chloroquine it was necessary to use large doses and to time them carefully.

In spite of the difficulties of the test, it is felt that it is a useful procedure for the investigation of selected drugs.

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CHOLINE ACETYLASE ACTIVITY IN THE ATRIA AND ITS POSSIBLE RELATION TO THE MAINTENANCE OF THE MEMBRANE POTENTIAL

BY

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Choline acetylase is present in rabbit atria and forms acetylcholine continuously. It is known that the contractions of isolated atria cease when the temperature falls to a point below 20° C., and that they can be started again by acetylcholine, which causes a rise in the transmembrane potential. The question may therefore be asked whether the transmembrane potential is normally kept sufficiently high for impulses to be propagated by the acetylcholine which is formed within the atria. The effect of lowering the temperature on choline acetylase activity has therefore been studied. The activity was found to decline much more steeply below 20° C. than between 33° and 20°. The Q_{10} rose to 7.8. This finding is consistent with the view that one function of choline acetylase activity is to maintain the transmembrane potential.

Studies on the isolated atria of the rabbit heart have shown that, as a result of cooling, the contractions are arrested and can then be restarted by the addition of acetylcholine (Marshall and Vaughan Williams, 1956). There is evidence that acetylcholine acts by raising the membrane potential (Marshall, 1957). Acetylcholine can be extracted from atria, and choline acetylase and cholinesterase are present in them. That the choline acetylase is not inactive, and that it is constantly forming acetylcholine, was shown by Burn and Kottegoda (1953), who found that eserine in concentrations from 10^{-6} to 10^{-5} g./ml. slowed the rate of contraction. Day (1956) found that the isolated rabbit heart perfused with Locke solution liberated acetylcholine into the perfusion fluid. The suggestion has therefore arisen that one of the functions of acetylcholine thus formed is to maintain the membrane potential at sufficient height and so to make possible the propagation of the impulses from the pacemaker.

Since cooling the atria to a temperature below 20° causes arrest of the atrial contractions, we have investigated the effect of cooling on the synthesis of acetylcholine to see if the synthesis declined at the point at which the contractions stopped.

METHODS

The methods used were similar to those previously described (Milton, 1959a, b).

Enzyme Preparation.—Acetone powders of rabbit atria which had been minced were prepared as follows. After washing the tissue in a mortar with a large volume (approx. 300 ml.) of cold dry acetone (−15°) which was rapidly poured off through a Buchner filter funnel, the tissue was ground up with several further additions of acetone until all the material had been transferred to the Buchner filter in the form of a fine powder. After drying on the filter paper for 5 min. the powder was transferred to a desiccator and evacuated over P_2O_5. After standing at 4° for 24 hr., the powder was removed from the desiccator, weighed and suspended (40 mg./ml.) in cysteine saline solution (6 mg. of neutralized cysteine hydrochloride/ml. 0.9% NaCl).

The solution was stored at −15° (for a minimum period of 24 hr.). Just before use it was thawed and centrifuged at 3,000 rev./min.

Incubation System. — The incubation system contained the following reagents (Hebb, personal communication). Co-enzyme A, (400 units/ml.), 0.05 ml.; 30% KCl, 0.04 ml.; 4% choline chloride, 0.04 ml.; 1.2% $MgCl_2.6H_2O$ 0.08 ml.; acetyl phosphate (10 mg./ml.), 0.14 ml.; (−)-cysteine HCl (neutralized; 30 mg./ml.), 0.12 ml.; phosphotransacetylase (2.5 mg./ml.), 0.10 ml.; eserine sulphate (1 mg./ml. in 4% NaCl), 0.10 ml.; water, 0.23 ml. to a total volume of 0.90 ml.

The co-enzyme A preparation was diluted with distilled water in those experiments in which different concentrations were required [0.05 ml. of co-enzyme A (400 units/ml.)=5 units enzyme/mg. of atrial acetone powder]. It was prepared by a modification of the method described by Hebb (1955). Acetyl

phosphate obtained commercially was suspended in distilled water. Phospho-transacetylase, which was also supplied by the Worthington Biochemical Corporation, U.S.A., was suspended in 0.02 N-$KHCO_3$.

The water used for making up solutions was obtained by glass distillation of tap water which had previously been demineralized using a Permutit demineralizing plant.

The reagents listed above were pipetted into suitable glass tubes and incubated for 15 min., to initiate the formation of acetyl co-enzyme A.

After 15 min., 0.1 ml. of the enzyme preparation to be tested was added to the incubation mixture. The tubes were corked and the incubation then allowed to proceed for 1 hr. As controls, similar tubes were set up which contained the same reagents, and 0.1 ml. of boiled enzyme. These controls were used in the assay when matching the test solutions against standard acetylcholine solutions.

After exactly 1 hr., the reaction was stopped by adding 6 drops of B.D.H. Universal indicator and 0.2 ml. 0.33 N-HCl and 1.0 ml. of phosphate buffer M/15. Frog Ringer solution (4 ml.) was added to each tube and the contents boiled for 1 min. After cooling, the volume was made up to 10 ml. with frog Ringer solution and stored at −15°.

The acetylcholine content was then assayed on the isolated frog rectus abdominis muscle preparation. The standard acetylcholine solutions were made up with addition of the control solutions in amounts corresponding to the amounts of test solution used.

Results

Temperature and Choline Acetylase Activity.—The results of five experiments to determine the effect of temperature on the formation of acetylcholine by choline acetylase present in acetone-dried powders from rabbit atria are given in Table I. For comparison, the results in each experiment were expressed in relation to the activity at 33° taken as 100, and the values so obtained are shown in Table II. The mean results showed that at 21° the activity was 43% of that at 33°, but that at 17° it had fallen to 21%, and at 13° to 9%. Thus the fall in activity was steep below 21°.

From these results the Q_{10} was calculated for each rise of temperature, and the values are shown in Table III, together with values for rabbit brain calculated from work of Milton (1958). Normal values for the Q_{10} of biological reactions are from 1.0 to 2.5, and this held for choline acetylase activity between 21° and 41°. Below 21°, however, the Q_{10} values steadily increased, being 6.2 between 17° and 21°, and 7.8 between 13° and 17°. The estimates for choline acetylase in atria were very similar to those in brain at corresponding temperatures.

TABLE I

THE EFFECT OF TEMPERATURE ON THE SYNTHESIS OF ACETYLCHOLINE BY RABBIT ATRIA

The values show the amount of acetylcholine synthesized in μg./g. of acetone powder/hr.

Temp. (°C.)	Expt. No.				
	1	2	3	4	5
13	—	5	38	19	31
17	—	50	78	62	44
21	125	88	138	81	100
25	131	137	169	119	138
29	175	206	288	150	187
33	238	231	350	194	238
37	300	—	—	—	—
41	323	—	—	—	—

TABLE II

CHOLINE ACETYLASE ACTIVITY

The results are expressed in relation to those at 33° taken as being 100.

Temp. (°C.)	Expt. No.					Mean
	1	2	3	4	5	
13	—	2·2	10·9	9·8	13·0	9·0
17	—	21·6	22·3	19·5	18·5	20·5
21	52·5	38·1	39·4	41·8	42·0	42·6
25	55·0	59·3	48·3	61·3	58·0	56·4
29	73·5	89·2	82·3	77·3	78·6	78·6
33	100	100	100	100	100	100
37	126	—	—	—	—	126
41	136	—	—	—	—	136

TABLE III

THE Q_{10} OF CHOLINE ACETYLASE FROM RABBIT ATRIA COMPARED WITH VALUES FOR CHOLINE ACETYLASE OBTAINED FROM RABBIT BRAIN

Temp. (°C.)	Atria	Temp. (°C.)	Brain
13–17	7·8		
17–21	6·2	17–22	6·7
21–25	2·0	22–27	3·1
25–29	2·3		
29–33	1·8	32–37	1·6
33–37	1·8		
37–41	0·7		

Effect of Acetylcholine on Synthesis in Cooled Atria.—The effect of acetylcholine on synthesis in atria which had been arrested by cooling was also studied. This was done because Bülbring and Burn (1949) found that, when an acetone powder was prepared from atria which had ceased to beat after many hours in the bath, the addition of acetylcholine to the powder increased the rate of acetylcholine synthesis. These findings have been confirmed by Milton (1959b). The first experiments were carried out using amounts of co-enzyme A which varied from 0 to 2.5 units/mg. of powder, and synthesis was compared in the presence of 300 μg. of acetylcholine/g. powder with synthesis in its absence. The results are shown in Table IV. It will be seen that the added acetylcholine had an inhibitory effect which decreased as the co-enzyme A concentration increased.

TABLE IV
THE RELATION OF CO-ENZYME A CONCENTRATION TO THE INHIBITORY ACTION OF ACETYLCHOLINE ON A CHOLINE ACETYLASE PREPARATION FROM ATRIA STOPPED BY COOLING

Co-A, co-enzyme A; ACh, acetylcholine; temperature 37°.

Co-A (units/ mg. Powder)	ACh Added (μg./g. Powder)	ACh Synthesized (μg./g./hr.)	Change
0	0	100	—
0	300	50	−50%
0·625	0	222	—
0·625	300	175	−21%
1·25	0	317	—
1·25	300	275	−13%
2·50	0	375	—
2·50	300	325	−13%

In the next experiments the amount of co-enzyme A was kept constant but low at 0.625 unit/mg. of powder, while the acetylcholine concentration was varied. The results in Table V show that the inhibitory action was proportional to the amount of acetylcholine added. When large amounts of co-enzyme A were used (5 units/mg.) the synthesis was practically unaffected by acetylcholine. Finally, experiments were done in which powder from cooled atria was incubated at 15° instead of at 37°. Again acetylcholine caused inhibition of synthesis in the absence of co-enzyme A.

Choline Acetylase Activity of Rabbit Ventricle.—Acetone-dried powders of rabbit ventricle were prepared and the choline acetylase activity was measured. For comparison duplicate tubes were set up containing acetone powders prepared from rabbit atria. The incubation was carried out at 37° and the results are shown in Table VI. The synthesis in ventricle was very small indeed.

Effect of Atropine.—If the acetylcholine formed in the atria maintains the transmembrane potential, it might be thought that atropine should abolish the potential. We carried out experiments in which atria were cooled until they ceased to beat, and then observed the restarting of the beat

TABLE V
RELATION BETWEEN AMOUNT OF ACETYLCHOLINE ADDED AND INHIBITION OF SYNTHESIS IN ATRIA. STOPPED BY COOLING

Concentration of co-enzyme A, 0·625 unit/mg. Incubation at 37°.

Acetylcholine Added (μg./g. Powder)	Acetylcholine Synthesized (μg./g./hr.)	% Change
0	158	—
12·5	154	−2·6
31·2	144	−9·2
62·5	117	−26·4
125·0	54	−65·8

TABLE VI
CHOLINE ACETYLASE ACTIVITY OF RABBIT VENTRICLE COMPARED WITH THAT OF RABBIT ATRIA

	Acetylcholine Synthesized μg./g. Acetone Powder/hr.	
	Ventricle	Atria
	19	275
	12·5	232
	44	300
Mean	25	269

on gradual warming. The addition of atropine sulphate in concentrations up to 10^{-5} g./ml. had no effect on the restarting of the beat.

DISCUSSION

In three different conditions acetylcholine has been found to restart the contractions of the atria. Marshall and Vaughan Williams (1956) found that, when the atria were cooled, the contractions ceased at a temperature below 20°. In most experiments they were still able to record action potentials at the pacemaker and, when these were present, they found that the addition of acetylcholine caused contractions to begin again. Marshall (1957) found that the process of cooling caused a fall in the transmembrane potential and that contractions ceased when it became less than 60 mV. She showed that the addition of acetylcholine caused a rise in the potential and that the restarting of the contractions was due to this rise. The action of acetylcholine is probably due to its effect in increasing the permeability of the membrane to potassium (Harris and Hutter, 1956) and so allowing the transmembrane potential to move nearer to the potassium equilibrium potential. At the higher potential an impulse is able to depolarize because the rate of entry of sodium ions is faster; as Weidmann (1955) has shown, when the transmembrane potential is too low, the rate of sodium entry is too slow to depolarize.

An earlier observation was that of Bülbring and Burn (1949), who allowed atria to contract until they stopped after being left in the organ bath for 30 to 40 hr. They showed that acetylcholine restarted the beat, and this result was confirmed by Holtz and Westerman (1955). Goodford (1959) determined the intracellular potassium and found that, when contractions stopped, it had fallen to a point at which the transmembrane potential could not have been more than 60 mV. The restarting of the contractions by acetylcholine could therefore be attributed to a rise in the membrane potential.

Later Briscoe and Burn (1954) found that atrial contractions were also arrested by quinidine,

and that again acetylcholine would restart them. When the contractions were arrested by quinidine, Armitage (1957) found that they could also be started again by reducing the external potassium concentration to one-half. This indicated that the arrest by quinidine was due to a fall in the membrane potential, and made it likely that the restarting by acetylcholine was due to the rise in the membrane potential.

Thus in the three conditions of atrial arrest there is good reason to believe that the action of acetylcholine in restarting the beat was due to the rise in membrane potential which it caused. The choline acetylase system in the atria can synthesize acetylcholine, and, as already stated, there is clear evidence that it is constantly being formed. The acetylcholine so formed could then have as one of its functions the maintenance of a normal transmembrane potential, and, if this were so, a fall in temperature which causes atrial arrest should cause a fall in acetylcholine synthesis.

We found that the activity of the choline acetylase at 21° was still about 40% of that at 33°. Below 21°, however, the activity fell very rapidly, being down to 20% at 17°, and down to 10% at 13°. Thus in the temperature range in which atria ceased to beat the choline acetylase activity was greatly affected by temperature. Whereas in the range from 33° to 21° the Q_{10} was 2.0, in the range from 21° to 17° it was 6.2 and from 17° to 13° it was 7.8. These observations support the view that the arrest of the atria by cooling is due to the reduction of choline acetylase activity, as a result of which insufficient acetylcholine is formed to maintain the transmembrane potential above 60 mV.

When atria arrested by cooling were restarted by the addition of acetylcholine to the bath, atropine abolished the effect (Marshall and Vaughan Williams, 1956). Similarly when they were restarted by vagal stimulation (Burn and Rand, 1958) atropine also abolished the effect. The restarting of atria by warming was not affected by atropine, and hence it might be concluded that warming did not restart the atria by increasing acetylcholine synthesis. However, it is quite possible that acetylcholine produced within the atria is acting at a point where it is inaccessible to the blocking action of atropine. Thus it is well known that certain parasympathetic effects, those of the vagus and the pelvic nerves on the muscular walls of the small and of the large intestine and of the bladder, are highly resistant to atropine. It is possible that studies with the electronmicroscope will throw more light on the location of the structures containing choline acetylase.

In the ventricles, choline acetylase activity was found to be negligibly small, from which it may be concluded that acetylcholine can exert no similar control over the membrane potential in ventricular muscle. The difference between atria and ventricles in this respect is parallel to the difference in the effect of acetylcholine on the action potential. In the atria, acetylcholine shortens the action potential whereas in the ventricles it has no effect.

If the conclusion concerning the function of acetylcholine formed in the atria is correct, we may have a clue to the reason for the formation of acetylcholine in the gill plates of *Mytilus edulis* where it maintains ciliary movement. It is possible that here also acetylcholine maintains the membrane potential sufficiently high for the impulses to be propagated which give rise to ciliary contraction.

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THE EFFECT OF PIPERAZINE ON SUCCINATE PRODUCTION BY *ASCARIS LUMBRICOIDES*

BY

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Piperazine reduced the production of succinate by *Ascaris lumbricoides*. This effect was reversible. There was a close parallelism between the concentrations of piperazine which paralysed the worm and those which inhibited the formation of succinate. Piperazine did not affect the incorporation of [2-^{14}C]lactate into succinate by strips of *Ascaris* muscle. It was concluded that production of succinate supplies energy for the contraction of *Ascaris* muscle.

Succinic acid has been identified in the perienteric fluid of *Ascaris lumbricoides* (*var. suis*) (Bueding and Farrow, 1956). In the muscle of this parasite at least two active enzymatic mechanisms are available which can account for the utilization of succinate: (*a*) the succinic oxidase system for the formation of fumarate (Bueding and Charms, 1952; Bueding, Entner, and Farber, 1955), followed by its conversion to pyruvate catalysed by fumarase and a "malic" enzyme (Saz and Hubbard, 1957); (*b*) decarboxylation of succinate to propionate (Saz and Vidrine, 1959). The presence of these metabolic systems concerned with the utilization of succinate together with the high concentration of succinate in the body fluid of *Ascaris* (Bueding and Farrow, 1956) suggest that succinate is very rapidly formed. The present paper reports that production of succinate by intact *Ascaris* can be demonstrated readily, a process inhibited reversibly by piperazine. This compound produces paralysis of the worm (Brown, Chan, and Hussey, 1955; Swartzwelder, Miller, and Sappenfield, 1955), and is effective in the treatment of ascariasis (see review by Bueding and Swartzwelder, 1957).

METHODS

The parasites were incubated for 24 hr. at 38° in Erlenmeyer flasks with a modified Ringer-Baldwin medium containing NaCl, 8.0 g.; KCl, 200 mg.; $CaCl_2$, 200 mg.; $MgSO_4$, 400 mg.; $NaHCO_3$, 1.8 g.; 2N-NaOH, 1 ml.; glucose, 2 g.; sulphafurazole, 500 mg.; penicillin, 100 mg.; streptomycin, 75 mg.; nystatin (Mycostatin), 10 mg.; distilled water, 1 l. While in earlier studies the addition of a sulphonamide, penicillin and streptomycin to the medium eliminated contamination with micro-organisms (Epps, Weiner, and Bueding, 1950), in the present experiments fungi grew not infrequently unless nystatin was present. This compound had no effect on the production of succinate by *Ascaris* in the concentration used. The worms were blotted and weighed before their transfer into the incubation medium. Each flask contained three worms in 150 ml. of medium.

The concentration of succinate in the worms was determined as follows. The parasites were homogenized in three times their weight of perchloric acid (5% v/w) and the precipitated proteins were separated by centrifugation at 3,000 rev./min. for 30 min.; a measured volume of the supernatant fluid was neutralized to *p*H 7.0 with 5N-KOH and its total volume recorded; to remove the insoluble potassium perchlorate the mixture was centrifuged (3,000 *g*; 30 min.). These operations were carried out between 2 and 4°. An aliquot of the supernatant was adjusted to *p*H 1.8 with 5N-H_2SO_4 and extracted with ether in a continuous extraction apparatus for 24 hr. The extract was evaporated to dryness, taken up in water, neutralized to *p*H 7.0 with 2N-KOH and the volume brought to 2.0 ml. with water; the succinate content of an aliquot of this neutralized extract was determined enzymatically by the method previously used (Bueding and Farrow, 1956). The succinate concentration of aliquots (usually 60 ml.) of the medium was analysed in the same manner, except that treatment with perchloric acid was omitted.

Stock solutions of piperazine were prepared by adjusting a concentrated solution of piperazine hexahydrate (40%) with 2N-HCl to *p*H 7.4. The incubation medium was used for further dilutions. All concentrations of piperazine are expressed in terms of the weight of the free base.

Ascaris muscle strips were obtained as described by Waters (1954) and incubated with [2-^{14}C]lactate in

a conventional Warburg apparatus. At the end of the incubation period, enzymatic reactions were stopped by placing the incubation mixture in a boiling-water bath for 10 min. After steam distillation, ether extraction and chromatography from Celite, succinate and lactate were isolated (Saz and Vidrine, 1959). The succinate fraction thus obtained was treated with $KMnO_4$ (Friedemann and Graeser, 1933) to oxidize possible contaminating acids. The succinate was re-isolated by ether extraction and again chromatographed from Celite. Succinate was degraded by oxidizing completely an aliquot of the sample to CO_2 (Van Slyke and Folch, 1940). Samples of $^{14}CO_2$ were precipitated as $Ba^{14}CO_3$ and the precipitate was collected as a finite layer on filter paper discs. Samples thus obtained were assayed for radioactivity with an ultra-thin window gas-flow counter.

TABLE I

PRODUCTION OF SUCCINATE BY *A. LUMBRICOIDES VAR. SUIS*

The worms were incubated for 24 hr. at 38° in a modified Ringer-Baldwin medium.

Expt. No.		Succinate Content (μM/10 g. of Worms)			
		Worms	Medium	Total	Change
1	Before incubation	62		62	
	After ,,	48	52	100	+38
2	Before ,,	55		55	
	After ,,	44	65	109	+54
3	Before ,,	55		55	
	After ,,	48	45	93	+38
4	Before ,,	66		66	
	After ,,	72	47	119	+43

TABLE II

EFFECT OF PIPERAZINE ON SUCCINATE PRODUCTION BY *A. LUMBRICOIDES*

Incubation period, 24 hr. Temperature: 38°.

Expt. No.	Piperazine (%)	Succinate Content					
		Worms		Medium		Total	
		(μM/10 g. of Worms)	Change (%)	(μM/10 g. of Worms)	Change (%)	(μM/10 g. of Worms)	Change (%)
1	—	74		38		112	
	0·1	50	−32	2	−95	52	−54
2	—	66		28		94	
	0·1	43	−35	4	−86	47	−50
3	—	86		67		153	
	0·1	68	−21	6	−91	74	−52
4	—	52		58		110	
	0·05	34	−35	5	−91	39	−64
5	—	55		64		119	
	0·05	36	−35	5	−92	41	−65
6	—	69		47		116	
	0·05	48	−31	7	−85	55	−57

RESULTS

Production of succinate by *Ascaris* was demonstrated readily by determination of succinate in the worms and in the incubation medium (Table I). While the succinate concentration of the worms did not change greatly during incubation, large quantities of succinate appeared in the medium; thus, the total amount of succinate found in the worm and in the medium was considerably greater than that present in the parasite before incubation.

While trying to see if piperazine produced any biochemical changes in *Ascaris*, it was found that this anthelmintic markedly reduced the production of succinate (Table II). After incubation with paralysing concentrations of piperazine, the succinate content of the worms was decreased significantly; the reduction in the amount of succinate released into the medium was even more pronounced. Succinate production was inhibited by piperazine to the same extent when glucose was omitted from the medium. This effect of piperazine was reversible: when, after incubation with piperazine, the worms were transferred into piperazine-free medium the total amount of succinate was about the same as in controls not incubated previously with piperazine (Table III).

Fig. 1 illustrates the relationship between the concentration of piperazine and the reduction in the succinate content of the medium. There was a good correlation between the paralysing action of piperazine and its inhibition of succinate production. When paralysis was complete, succinate production was strongly inhibited.

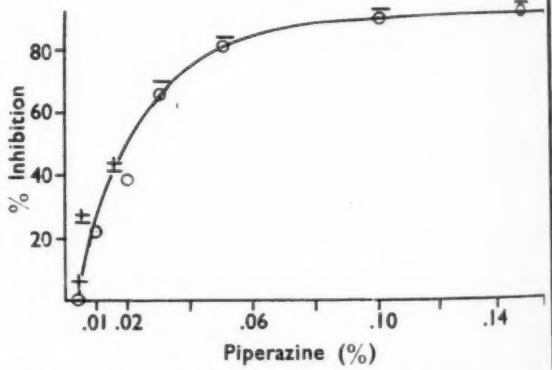

FIG. 1.—The relation between concentration of piperazine and inhibition of succinate production. −, Non-motile; ±, barely motile; +, motile.

Concentrations of piperazine which produced incomplete, yet clearly noticeable, reduction in motility inhibited succinate production appreciably but to a lesser extent. Piperazine in a concentration which did not affect motility did not alter succinate production.

The action of piperazine on succinate production differs in some respects from that of the phenothiazine derivative promethazine (Table IV). In the first place, succinate production was inhibited to a lesser degree than with piperazine. Furthermore, inhibition of succinate production not only persisted but became much more pronounced after the worms had been transferred into medium free of promethazine. Therefore, in contrast to piperazine, promethazine has an irreversible effect on succinate production.

Ascaris muscle strips incorporate [2-^{14}C]lactate into succinate (Saz and Vidrine, 1959). The effect of piperazine upon the incorporation was studied to determine whether paralysis results from an impaired rate of formation of succinate, or *vice versa*. Six worms were incubated in each of two flasks containing the glucose-salt-antibiotic mixture, one with 0.1% piperazine as well. After 24 hr., muscle strips were obtained from both piperazine-treated and untreated worms and incorporation of [2-^{14}C]lactate into succinate was determined. Piperazine (0.1%) was added only to the incubation mixture which contained the muscle strips from piperazine-treated worms. Results of these experiments are recorded in Table V. It can be seen that piperazine had essentially no effect upon the incorporation of [2-^{14}C]lactate

TABLE III

REVERSIBILITY OF THE INHIBITORY EFFECT OF PIPERAZINE ON SUCCINATE PRODUCTION IN *A. LUMBRICOIDES*

After the first incubation period (24 hr.; 38°) the worms were transferred into piperazine-free control media and incubated for another 24 hr.

Expt. No.	Piperazine (%)	First Incubation Period						Second Incubation Period					
		Succinate Content						Succinate Content					
		Worms		Medium		Total		Worms		Medium		Total	
		(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)
1	—	55		64		119		61		72		133	
	0·05	36	−35	5	−92	41	−65	79	+30	62	−14	141	+6
2	—	66		28		94		45		73		118	
	0·1	43	−35	4	−86	47	−50	58	+29	58	−20	116	−2
3	—	78		48		126		66		42		108	
	0·05	43	−45	6	−88	49	−61	61	−8	37	−12	98	−10

TABLE IV

IRREVERSIBILITY OF THE INHIBITORY EFFECT OF PROMETHAZINE ON SUCCINATE PRODUCTION IN *A. LUMBRICOIDES*

After the first incubation period (24 hr.; 38°) the worms were transferred into promethazine-free control media and incubated for another 24 hr.

Expt. No.	Promethazine (%)	First Incubation Period						Second Incubation Period					
		Succinate Content						Succinate Content					
		Worms		Medium		Total		Worms		Medium		Total	
		(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)	(μM/ 10 g. of Worms)	Change (%)
1	—	82		47		129		72		42		114	
	0·004	74	−10	38	−19	112	−13	44	−39	5	−88	49	−57
2	—	60		41		101		53		35		88	
	0·004	48	−20	28	−32	76	−25	22	−59	0	−100	22	−75
3	—	47		49		96		43		38		81	
	0·004	41	−13	29	−41	70	−27	21	−51	4	−89	25	−69

TABLE V

EFFECT OF PIPERAZINE UPON THE INCORPORATION OF [2-^{14}C]LACTATE INTO SUCCINATE BY MUSCLE STRIPS OF *A. LUMBRICOIDES*

Worms were incubated in the modified Baldwin-Ringer medium with or without piperazine (0.1%). After 24 hr., muscle strips were obtained from six piperazine-treated and six control worms. Each set of strips was added to a Warburg vessel which contained 150 μmole of lithium [2-^{14}C]lactate (count/min. = 49,760/μmole), 0·1% piperazine where indicated and antibiotic-free medium. Vessels were shaken for 100 min. at 37° ; the gas phase was 95% N_2 and 5% CO_2. The weight of muscle in each flask was 7 to 8 g.

Expt. No.	Piperazine	Lactate Disappearance (μmole)	Succinate Content				
			Recovered	Total Radioactivity		Specific Radioactivity	
			(μmole)	(Count/min.)	Change (%)	Count/min./μmole	Change (%)
1	−	65·2	55·3	184,700		3,340	
	+	56·2	54·9	158,660	−14·1	2,890	−13·5
2	−	−	44·4	144,740		3,260	
	+	−	39·4	147,360	+1·8	3,740	+14·7

into succinate. There was a slight decrease in the specific and total radioactivities in Experiment No. 1, and a slight increase in Experiment No. 2. As these are considered to be within the range of experimental variation piperazine has no effect upon the conversion of lactate to succinate.

DISCUSSION

Norton and de Beer (1957) have reported that piperazine blocks the response of *Ascaris* muscle to stimulation by acetylcholine ; the concentration/response curve obtained by these authors is very similar to that relating concentration of piperazine to inhibition of succinate production (Fig. 1). Furthermore, both the neuromuscular blocking (Norton and de Beer, 1957) and metabolic effects of piperazine are reversible. Therefore, the question arises whether and in what manner these two actions of piperazine on *Ascaris* are related. The paralysis may either be the cause or the result of the reduced rate of formation of succinate. It is conceivable that, at the neuromuscular junction, chemical reactions associated with succinate production may be required for the response of *Ascaris* muscle to acetylcholine. If this were so, piperazine should inhibit succinate production in isolated muscle strips from *Ascaris*. However, paralysing concentrations of piperazine do not affect appreciably the rate of incorporation of [2-^{14}C]lactate into succinate. Therefore, it is concluded that the inhibition of succinate production in *Ascaris* by piperazine is the result of myoneural block. In skeletal muscle of vertebrates the energy from the anaerobic conversion of carbohydrate to lactic acid is utilized ultimately for muscular contraction through resynthesis of energy-rich phosphate compounds. In *Ascaris*, reactions involved in the formation of succinate may be concerned with the generation of energy for muscular contraction ; if this were so, reduction or complete suppression of muscular activity by piperazine would lower the energy requirement of muscle and result in a decreased formation of succinate. In the light of the findings of Norton and de Beer (1957) the observations reported here indicate that production of succinate plays a rôle in the chemistry of muscular contraction of *Ascaris*.

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THE INHIBITION OF AMINE OXIDASE AND THE CENTRAL STIMULATING ACTION OF THE STEREOISOMERIC AMPHETAMINES AND 1-PHENYLETHYLAMINES

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The stereoisomers of amphetamine and 1-phenylethylamine have been studied in the rat both as central stimulants and as inhibitors of amine oxidase from brain, liver, and kidney. There was no correlation between these two effects; thus it is unlikely that the central stimulating action of amphetamine is due to inhibition of amine oxidase.

It is known that species differences exist in the pattern of substrate and inhibitor specificities Pratesi and Blaschko (1959) showed that, for a substrate as well as for an inhibitor, the presence of a centre of asymmetry in the molecule may confer different affinities (or rates of oxidation) on the two enantiomorphs, and also that this depends on the source of the enzyme.

Mann and Quastel (1940) suggested that central stimulation by amphetamine might be due to inhibition of amine oxidase. However, although in rabbit and man (+)-amphetamine (dexamphetamine) is a stronger stimulant than the (−) form, Pratesi and Blaschko (1959) have shown that the two stereoisomers are equally active as inhibitors of rabbit liver enzyme.

To test the idea that amphetamine owes its analeptic properties to its inhibitory action on amine oxidase, it was clearly necessary to use amine oxidase from the same species as that used for the pharmacological experiments. It was also desirable to use amine oxidase from the brain in preference to liver, because the patterns of substrate and inhibitor specificities might vary in different organs.

The present paper describes studies in the rat on dexamphetamine and (−)-amphetamine and (+)- and (−)-1-phenylethylamine as central stimulants and as inhibitors of amine oxidase from different organs (brain, liver, kidney). We studied 1-phenylethylamine because Pratesi and Blaschko (1959) found it to be a stronger inhibitor of the rabbit and cat liver enzyme than amphetamine; moreover 1-phenylethylamine had not yet been studied as a central stimulant. We have also studied the activity of dexamphetamine and (−)-amphetamine on amine oxidase from rabbit brain.

METHODS

The two amphetamines and 1-phenylethylamines used in our experiments were prepared by Dr. A. La Manna of our Institute. All these substances were obtained as the hydrochlorides. The signs (+) and (−) refer to the rotation of the hydrochlorides in aqueous solution. In some pharmacological experiments we have also used the sulphate of dexamphetamine.

Toxicity was studied in mice weighing 20 to 25 g. by injecting aqueous solution of the substances intraperitoneally. The animals were placed in groups of two or three in glass cages and observed for 24 hr.

Central stimulant activity was studied by measuring the duration of central depression in rats treated with chloral hydrate; the substance under examination was injected 5 min. after the beginning of the depression.

The sources of amine oxidase were rat liver, brain, and kidney, and rabbit brain. The fresh tissue was ground with sand; 2 ml. of 0.067 M-sodium phosphate buffer at *p*H 7.4 were added for each 1 g. of fresh tissue and the sand was then removed by centrifugation. Sometimes the rabbit brain was homogenized in a Potter's tube with twice its volume of buffer. The preparations were dialysed for 4.5 hr. against 0.067 M-sodium phosphate buffer and then overnight against tap water; one tenth volume of 0.2 M-sodium phosphate buffer was finally added.

Enzymatic activity was measured manometrically. The manometer flasks contained 2.0 ml. of fluid and 0.3 ml. of N-KOH. The temperature was 37° and the gas phase was O_2. Tyramine hydrochloride was always used as substrate at a final concentration of 0.01 M. The inhibitor was tipped from the side arm together with the substrate at the zero time.

RESULTS

Toxicity.—The results obtained by intraperitoneal administration of (+)- and (−)-1-phenylethylamine to mice are shown in Table I.

TABLE I

LETHAL EFFECTS OF (+)- AND (−)-1-PHENYLETHYLAMINE IN MICE

The drugs were injected intraperitoneally. The values represent number of mice killed and number of mice injected.

Drug	Dose			
	200 mg./kg.	250 mg./kg.	275 mg. kg.	300 mg./kg.
(+)-1-Phenylethylamine hydrochloride ..	0/4	0/6	4/6	5/6
(−)-1-Phenylethylamine hydrochloride ..	0/4	0/6	3/6	5/6

The acute toxicities of (+)- and of (−)-1-phenylethylamine are almost equal. With doses higher than 250 mg./kg. bodyweight, signs of motor excitement appeared, which intensified as the dose increased and which were more evident with (−)-1-phenylethylamine. Lethal doses caused death within a few minutes; with sublethal doses motor excitement subsided within a few hours.

Analeptic Action.—The 1-phenylethylamines and amphetamines were compared. The results are shown in Table II.

With chloral hydrate, the duration of central depression (the interval between the disappearance and reappearance of the righting reflex) was easy to determine. Differences with (+)- and (−)-1-phenylethylamine were not significant, whereas dexamphetamine was at least twice as active as (−)-amphetamine. (+)- and (−)-1-Phenylethylamine were five to six times less potent than dexamphetamine sulphate (at a dose level of 20 mg./kg.).

TABLE II

CENTRAL STIMULATING ACTION OF (+)- AND (−)-1-PHENYLETHYLAMINE AND OF DEXAMPHETAMINE AND (−)-AMPHETAMINE IN RATS

Chloral hydrate (300 mg./kg. intraperitoneally) was given in all experiments. All other drugs were injected subcutaneously. The dose of the drug marked with an asterisk has been calculated as if it were the sulphate. Narcosis time denotes the interval between the disappearance and the reappearance of the righting reflex.

Drug	Dose (mg./kg.)	No. of Rats	Narcosis Time	
			Mean and S.E. of Mean (min.)	% of Controls
Chloral hydrate alone ..		8	92±2·6	100
(−)-1-Phenylethylamine hydrochloride	100	6	68±5·9	73
(−)-1-Phenylethylamine hydrochloride	150	8	55±3·5	60
(+)-1-Phenylethylamine hydrochloride	100	6	75±3·5	81
(+)-1-Phenylethylamine hydrochloride	150	8	64±1·9	69
Dexamphetamine sulphate	20	8	67±2·0	72
Chloral hydrate alone ..		8	119±6·8	100
Dexamphetamine sulphate	20	7	56±2·5	48
,, ,,	10	7	97±7·3	81
(−)-Amphetamine hydrochloride*	40	9	66±7·0	55

Action on Amine Oxidase.—With enzyme preparations from rat liver, different inhibitor concentrations have been tested. The results obtained, expressed as the % inhibition of O_2 consumption 15 and 30 min. after the experiment had started, are shown in Table III.

TABLE III

INHIBITION OF AMINE OXIDASE FROM RAT LIVER

The substrate was 0·01 M-tyramine. Values are means of 3 to 5 experiments.

Inhibitor	Final Concentration (M)	% Inhibition of O_2 Uptake	
		After 15 min.	After 30 min.
Dexamphetamine ..	0·01	67	70
(−)-Amphetamine ..	0·01	52	51
(+)-1-Phenylethylamine	0·01	72	70
(−)-1-Phenylethylamine	0·01	24	30
Dexamphetamine ..	0·0033	52	52
(−)-Amphetamine ..	0·0033	31	32
(+)-1-Phenylethylamine	0·0033	53	51
(−)-1-Phenylethylamine	0·0033	12	15
Dexamphetamine ..	0·0017	46	47
(−)-Amphetamine ..	0·0017	23	26
(+)-1-Phenylethylamine	0·0017	46	44
(−)-1-Phenylethylamine	0·0017	5	8
Dexamphetamine ..	0·001	34	35
(−)-Amphetamine ..	0·001	20	22
(+)-1-Phenylethylamine	0·001	31	29
(−)-1-Phenylethylamine	0·001	2	2

Fig. 1 shows the time courses of oxidation using the four inhibitors at 0.01 M concentration. At this concentration dexamphetamine was more active than the (−) isomer; an equal inhibition (52%) was obtained with 0.01 M-(−)-amphetamine and 0.0033 M-dexamphetamine. (+)-1-Phenylethylamine was more active than (−)-1-phenylethylamine; the difference of activity between these two antipodes was even greater than for amphetamine because equal inhibitory effects (30%) were obtained with 0.01 M-(−)-1-phenylethylamine and 0.001 M-(+)-1-phenylethylamine. There were no differences between dexamphetamine and (+)-1-phenylethylamine in equimolecular concentrations.

The inhibitions after 15 and 30 min. were almost equal. Fig. 1 shows that, at least for the duration of our experiments, the relationship between O_2 consumption and time was linear, both in presence of substrate only and in the presence of both tyramine and inhibitor.

To study organ specificity, work on rat liver was extended to brain and kidney preparations. The inhibitors were used in two concentrations, 0.01 M and 0.001 M (Table IV). For enzymes obtained from brain and kidney we again observed that dexamphetamine was a stronger inhibitor than (−)-amphetamine, and that (+)-1-phenylethylamine was more active than (−)-1-phenylethylamine. With brain as well as with liver amine oxidase, dexamphetamine and (+)-1-

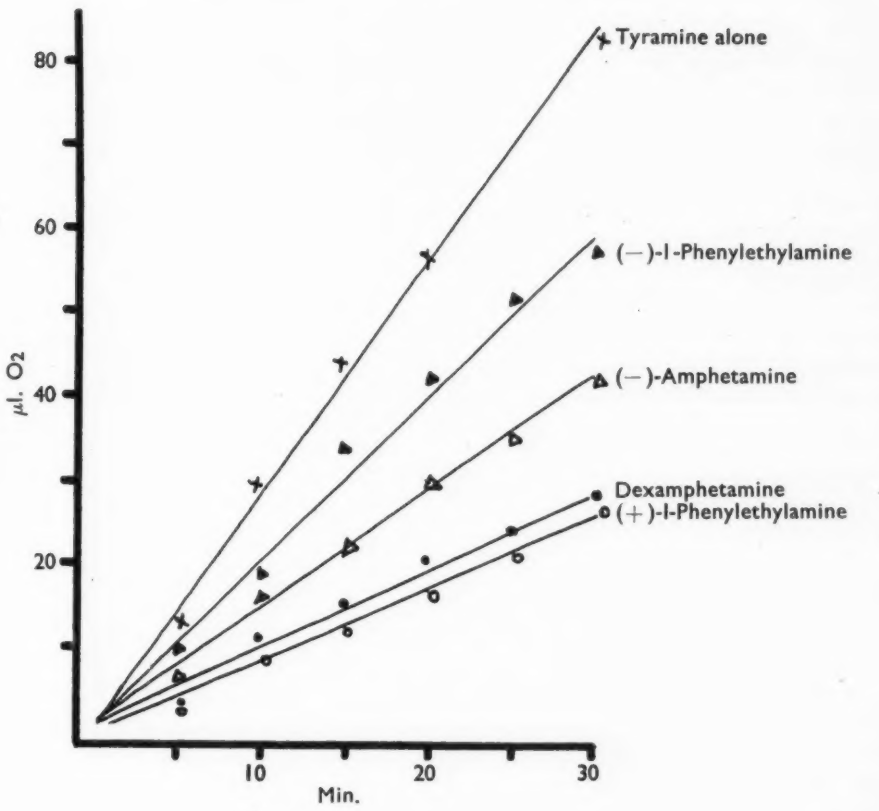

FIG. 1.—Inhibition of amine oxidase by dexamphetamine, (−)-amphetamine, and by (+)- and (−)-1-phenylethylamine (0.01 M). Rat liver was used as the source of enzyme, and tyramine as substrate. Ordinate, oxygen uptake; abscissa, time.

TABLE IV
INHIBITION OF AMINE OXIDASE FROM RAT LIVER, BRAIN, AND KIDNEY

Drug	Final Concentration (M)	% Inhibition of O_2 Uptake					
		Liver		Brain		Kidney	
		After 15 min.	After 30 min.	After 15 min.	After 30 min.	After 15 min.	After 30 min.
Dexamphetamine	0·01	67	70	71·5	69	79	83
(−)-Amphetamine	0·01	52	51	32	37·5	50	58
(+)-l-Phenylethylamine	0·01	72	70	74·5	73·5	58	62
(−)-l-Phenylethylamine	0·01	24	30	59	56·5	15	31
Dexamphetamine	0·001	34	35·5	39	39	34	41
(−)-Amphetamine	0·001	20	22	21	19	14	17
(+)-l-Phenylethylamine	0·001	31	29·5	28	26	22	22
(−)-l-Phenylethylamine	0·001	2	2	14	12	7	9

phenylethylamine were almost equally potent; with kidney amine oxidase dexamphetamine caused a slightly stronger inhibition.

In the experiments on the rabbit brain, only dexamphetamine and (−)-amphetamine were used as inhibitors. The results, expressed as % inhibition of O_2 consumption, were (average values): 0.01 M dexamphetamine, 80%; 0.01 M (−)-amphetamine, 66%; 0.001 M dexamphetamine, 36%; 0.001 M (−)-amphetamine, 22%.

On the basis of these results it follows that dexamphetamine was slightly more active than (−)-amphetamine.

DISCUSSION

To investigate whether inhibition of amine oxidase and central stimulation by amphetamine run parallel, both actions were studied in one and the same animal species using the enantiomorphic pairs of the two substances, amphetamine and 1-phenylethylamine. Reports of the action of 1-phenylethylamine are limited to the description of its pressor activity, which is about 1/1,000 that of adrenaline (Barger and Dale, 1910). Our experiments show that 1-phenylethylamine had pharmacological characteristics different from those of amphetamine.

The behaviour of the animals treated with 1-phenylethylamine was characterized by signs of

motor excitement only if lethal or nearly lethal doses were administered. The lethal dose was similar whether the animals were alone or in groups (J. H. Burn, personal communication). Spontaneous activity, however, was increased by doses of amphetamine well below lethal dose; the presence of other animals in the cage during the experiment greatly increased toxicity (Bovet and Bovet-Nitti, 1948).

The results of the biochemical experiments with rat liver as the source of amine oxidase were clearly at variance with those obtained in pharmacological experiments; dexamphetamine and (+)-1-phenylethylamine were equally active as amine oxidase inhibitors but differed widely in their central effects, while (+)- and (−)-1-phenylethylamine were equi-active pharmacologically but had very different activities as inhibitors of amine oxidase.

The results obtained with enzyme preparations from kidney and brain confirmed the conclusions reached with the enzyme from liver; they excluded organ specificity, at least within the range of the experiments carried out, and this extends observations by Blaschko and Himms (1955).

The results obtained with preparations of rabbit brain demonstrated a higher activity of dexamphetamine than of the (−) form, a difference of the same order as that found in the pharmacological experiments. Pratesi and Blaschko (1959), using rabbit liver as source of amine oxidase, found that the two stereoisomers of amphetamine were equally active as inhibitors. It is interesting that dexamphetamine, which in our experiments on rat liver proved to be as strong an inhibitor as (+)-1-phenylethylamine, appears to be more active on amine oxidase from guinea-pig liver than on that from rabbit liver (Pratesi and Blaschko, 1959). Such findings emphasize the dependence of stereospecificity on animal species.

A correlation between central stimulant action of amphetamine and inhibition of amine oxidase had been proposed by Mann and Quastel (1940) on the basis of the observation that the sequence of activity of amphetamine and three other phenylisopropylamines was the same for the effect on O_2 consumption of brain slices, for the anti-amine oxidase effect, and for the pharmacological action. Activity *in vivo* may be partly determined by unknown factors, but the lack of correlation between the inhibition of amine oxidase and the pharmacological effects of dexamphetamine and (+)-1-phenylethylamine and a similar lack of correlation between the two pairs of enantiomorphs can hardly be reconciled with the idea that central stimulation by amphetamine is due to its activity as an inhibitor of amine oxidase.

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ACTIONS OF DIMETHYLPHENYLPIPERAZINIUM

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The actions of 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) have been studied to discover under what conditions a blocking action could be seen. Dimethylphenylpiperazinium has a stimulant action on autonomic ganglia, stimulating the superior cervical ganglion and causing contraction of the nictitating membrane. It caused slowing followed by acceleration of the rate of beat of isolated rabbit atria. The denervated gastrocnemius muscle contracted if dimethylphenylpiperazinium was injected intra-arterially. Under other circumstances a blocking action was seen. It blocked peristalsis in the isolated guinea-pig ileum stimulated by raising intraluminal pressure and inhibited the response of the rat diaphragm and of the cat gastrocnemius stimulated through the motor nerve. It is suggested that dimethylphenylpiperazinium acts by depolarization, causing stimulation of resting muscle but inhibition by prolonging depolarization.

1,1 - Dimethyl - 4 - phenylpiperazinium iodide (DMPP) was first investigated by Chen, Portman, and Wickel (1951). It is primarily a hypertensive drug, the rise in blood pressure being due to stimulation of sympathetic ganglia and to the release of catechol amines from the suprarenal gland; it also has a stimulant effect upon plain muscle, like guinea-pig ileum, which is abolished by hexamethonium. It is therefore a "nicotine-like" drug, but unlike nicotine it appears to have little blocking action on ganglia, even in large doses. For this reason it has been used by Chen *et al.* (1951) and by Fakstorp and Pederson (1954) in preference to nicotine as an agonist in estimating the efficiency of ganglion-blocking agents on plain muscle preparations, such as guinea-pig ileum. Most ganglionic stimulators, like nicotine and tetramethylammonium, block ganglionic transmission in large doses or after repeated administration of small doses, so that dimethylphenylpiperazinium seemed to be an exceptional drug and worthy of further investigation. In particular my object was to discover under what conditions, if any, dimethylphenylpiperazinium would block ganglion cells.

Chen and Portman (1954) showed that dimethylphenylpiperazinium iodide caused a block of the peristaltic reflex of the guinea-pig ileum set up as described by Trendelenburg (1917). Leach (1957) confirmed this finding, and he also showed that, when a maintained contraction of the nictitating membrane of the cat was produced by stimulation of the preganglionic cervical sympathetic, dimethylphenylpiperazinium injected intra-arterially caused a lessening of the contraction, which then slowly returned.

The experiments reported here concern the effects of dimethylphenylpiperazinium on the spinal cat, on the isolated atria of the rabbit, on the peristaltic movement of guinea-pig intestine, on the skeletal neuromuscular junction (rat and cat), and upon the vessels of the hindleg of the dog.

METHODS

Spinal cats were prepared by the method described by Burn (1952). The contractions of the nictitating membrane were recorded by using a fine silk thread, attached to the tip of the membrane, which passed round a pulley and to an isotonic frontal writing lever having a magnification of about six times. The isolated atria of the rabbit were dissected clean and suspended in an isolated organ bath in Locke solution containing twice the ordinary amount of dextrose. Aeration was carried out with oxygen, and the beat was recorded by attaching a thread from the tip of one atria to a Starling heart lever. The guinea-pig ileum preparation was that described by Bülbring and Lin (1958) and Bülbring, Crema, and Saxby (1958). The rat diaphragm preparation was that of Bülbring (1946). The response of the gastrocnemius muscle of the spinal cat was recorded by detaching a portion of the os calcis into which the tendon of Achilles is inserted, and connecting it by a wire to an isometric tension lever. A rod passed through a hole drilled near the lower end of the femur was held rigidly between clamps. Electrodes were placed on the sciatic nerve which was stimulated by maximal single rectangular

wave shocks, duration of 0.7 msec. at a frequency of about 13/min. The hindleg of the dog was perfused with blood from a Dale-Schuster pump. A second pump perfused the lungs of another dog to provide oxygenation. Outflow was measured with the recorder of Stephenson (1949).

Results

Effects in the Spinal Cat.—Dimethylphenylpiperazinium iodide injected intravenously into a spinal cat caused a rise of blood pressure and a contraction of the nictitating membrane. In many instances, 10 or 11 times as much nicotine as dimethylphenylpiperazinium (in terms of base) was required to produce a similar rise in blood pressure and contraction of the nictitating membrane. When the suprarenal glands were excluded, the dose relationship between dimethylphenylpiperazinium and nicotine was of the order of one to three, a value which has been given by other workers using anaesthetized animals. However, when injections were made into the lingual artery the same amount of both drugs was required to produce similar contractions of the nictitating membrane. Fig. 1 shows the contractions of the nictitating membranes

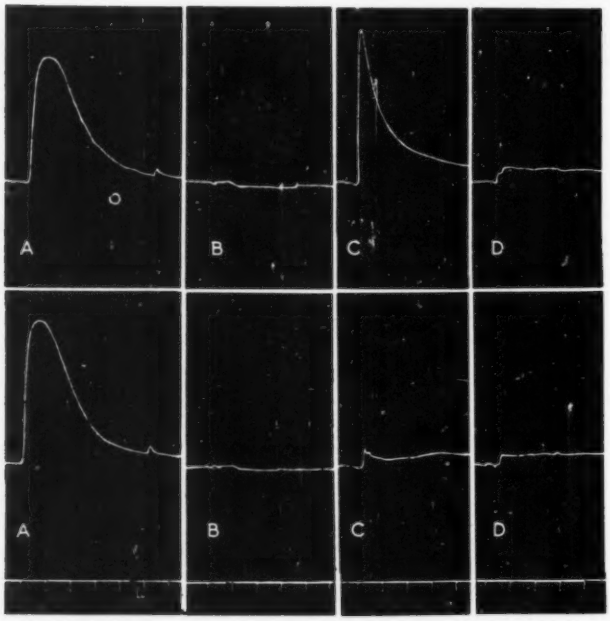


FIG. 1.—Spinal cat. Upper record, right nictitating membrane (preganglionic fibres cut). Lower record, left nictitating membrane (acutely denervated by removal of superior cervical ganglion). **A**, 80 μg. of dimethylphenylpiperazinium. Between **A** and **B**, the suprarenal glands were excluded from the circulation and the cat was eviscerated. **B**, 80 μg. of dimethylphenylpiperazinium. **C**, 500 μg. of dimethylphenylpiperazinium. Between **C** and **D**, 9.0 mg. of hexamethonium was given intravenously. **D**, 1.0 mg. of dimethylphenylpiperazinium. Time, 30 sec.

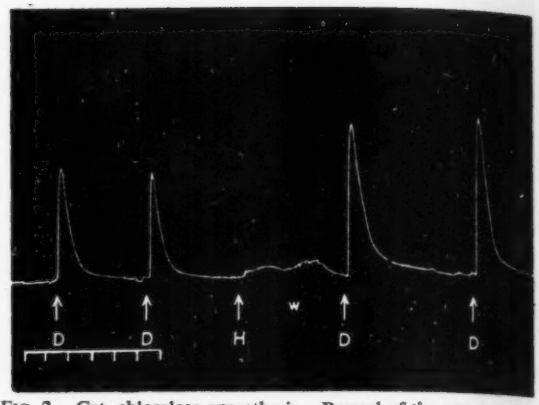
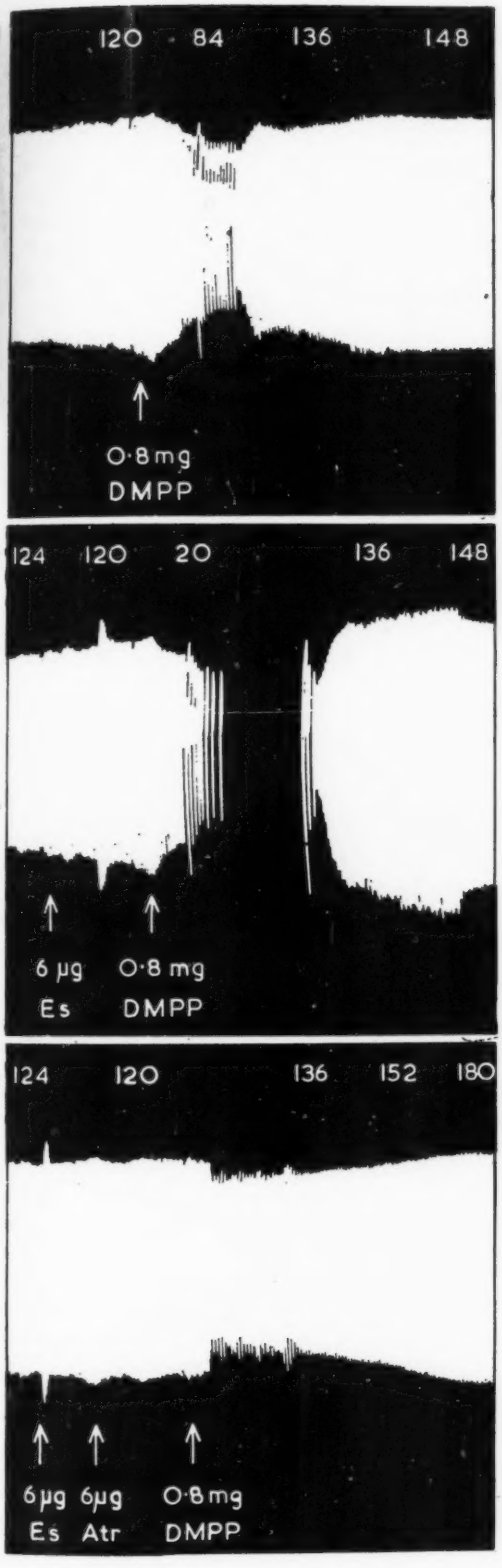


FIG. 2.—Cat, chloralose anaesthesia. Record of the response of the nictitating membrane with the preganglionic fibres cut. Injections given into the lingual artery with the external carotid artery occluded. **D**, 4 μg. of dimethylphenylpiperazinium. **H**, 100 μg. of histamine dihydrochloride. Time, 30 sec.

produced when dimethylphenylpiperazinium was injected intravenously into a spinal cat. The right membrane (upper record) was normal, but on the left side the superior cervical ganglion had been removed at the beginning of the experiment (lower record). At **A** 80 μg. of dimethylphenylpiperazinium iodide intravenously caused a contraction of both nictitating membranes. These contractions must have been due to liberation of catechol amines from the suprarenal glands, since after exclusion of the suprarenal glands and after evisceration the same dose of dimethylphenylpiperazinium had no effect (**B**). At **C** 500 μg. of dimethylphenylpiperazinium was injected and a contraction of the right membrane occurred, but there was no effect on the left side. When a total of 9.0 mg. of hexamethonium was given intravenously between **C** and **D**, dimethylphenylpiperazinium then had no effect. When dimethylphenylpiperazinium was injected into the lingual artery of a cat anaesthetized with chloralose, an injection of histamine intra-arterially potentiated the action of subsequent injections of dimethylphenylpiperazinium as shown in Fig. 2.

Effects on the Heart.—When added to a bath containing isolated atria of the rabbit, dimethylphenylpiperazinium iodide caused inhibition followed by stimulation, but after atropine stimulation only was seen. In Fig. 3*a*, 0.8 mg. of the iodide produced a slowing in rate followed by an acceleration, but, as shown in Fig. 3*b*, after 6 μg. of eserine, the slowing was greatly potentiated although the stimulant action which followed was still present. When 6 μg. of



atropine was added, the same dose of dimethylphenylpiperazinium failed to cause slowing, and the acceleration in rate which followed was greater (Fig. 3*c*). The stimulant action of dimethylphenylpiperazinium on isolated atria was abolished by hexamethonium and by ephedrine. Dimethylphenylpiperazinium, injected into the venous inflow of the Starling heart-lung preparation in which the coronary flow was recorded by means of a Morawitz cannula in the coronary sinus, caused increase in both systemic and coronary flow. A similar effect was produced by nicotine, both effects being abolished by hexamethonium.

Effects on Peristalsis.—The isolated guinea-pig ileum preparation of Bülbring and Lin (1958) was used, and the longitudinal movements, the intraluminal pressure and the flow were recorded. When dimethylphenylpiperazinium iodide (200 µg.) was injected into the fluid passing through the intestine, a lowering of the threshold of the intraluminal pressure occurred (white dots in Fig. 4*b*). There was a brief stimulation of the longitudinal movements, which was followed later by inhibition of the peristalsis. This lowering of the threshold of the intraluminal pressure was larger than when a similar dose of nicotine was given (Fig. 4*a*). When the drugs were added to the fluid in which the intestine was immersed, that is applied to the outside of the intestine, dimethylphenylpiperazinium (50 µg.) stopped peristalsis and flow as shown in the middle and lower record of Fig. 5*a*. Excitation, however, of the circular muscle occurred which can be seen in the uppermost record in Fig. 5*a*. Nicotine acid tartrate (50 µg.) caused complete paralysis without any excitation of the circular muscle (Fig. 5*b*).

Effects on the Skeletal Neuromuscular Junction.—Dimethylphenylpiperazinium added to the fluid bathing the isolated rat phrenic nerve diaphragm preparation caused a reduction in the height of the muscle twitch in response to single stimuli applied to the phrenic nerve. The effect produced by 0.5 mg. was greater than that produced by 6.0 mg. nicotine acid tartrate (Fig. 6). In the gastrocnemius muscle preparation of the cat, dimethyl-

FIG. 3.—Isolated rabbit atria suspended in Locke solution containing twice the normal dextrose concentration. Bath vol., 35 ml. (*a*) 0.8 mg. of dimethylphenylpiperazinium (DMPP) caused slowing of the rate followed by acceleration. (*b*) In the presence of 6 µg. of eserine, 0.8 mg. of dimethylphenylpiperazinium caused arrest, followed by acceleration. (*c*) When atropine (6 µg.) was given after eserine, 0.8 mg. of dimethylphenylpiperazinium caused only acceleration. The numerals above each record give beats/min.

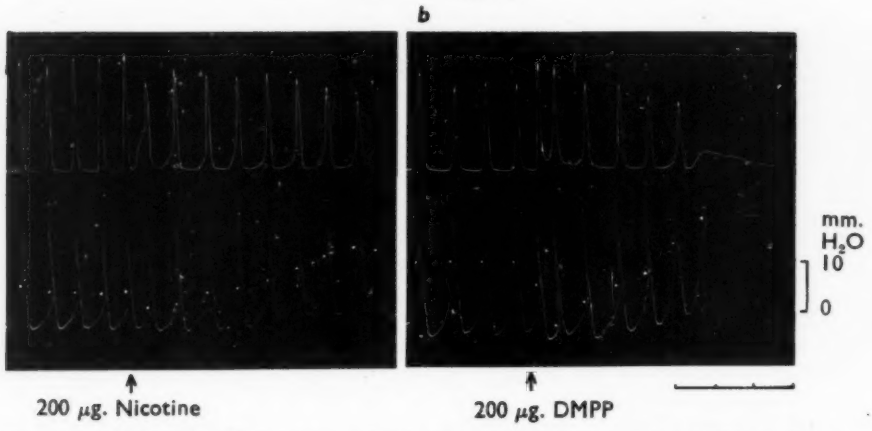


FIG. 4.—Isolated guinea-pig ileum suspended in Tyrode solution to record peristalsis by the method of Bülbring and Lin (1958). Upper record, contractions of the longitudinal muscle. Lower record, intraluminal pressure. (*a*) 200 μg. of nicotine acid tartrate injected into the lumen caused a transitory fall of the threshold of pressure (white dots) required to elicit the peristaltic reflex. (*b*) 200 μg. of dimethylphenylpiperazinium (DMPP) injected into the lumen caused a greater fall of threshold. This stimulation was followed by inhibition of peristalsis. Time, 1 min.

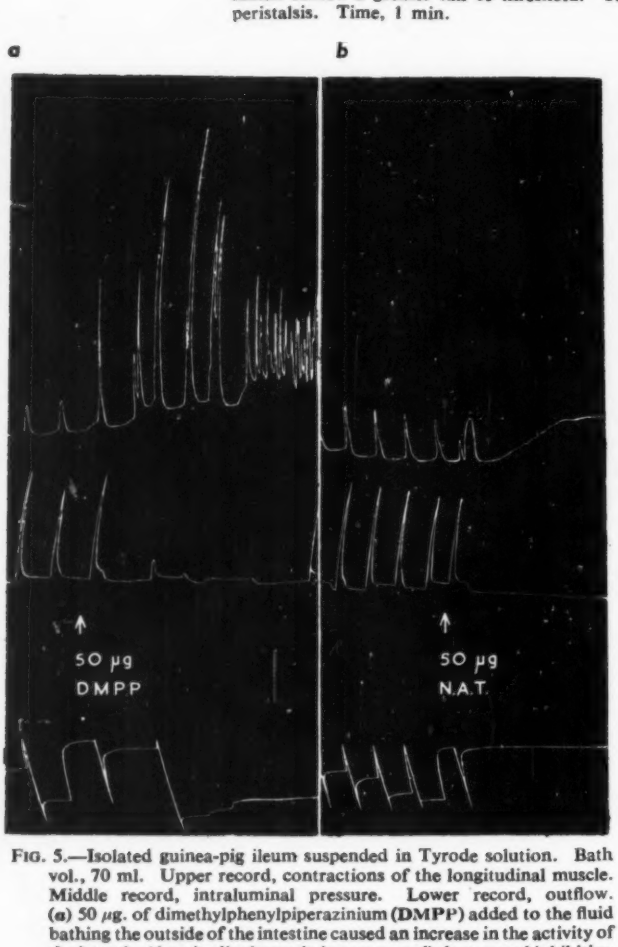


FIG. 5.—Isolated guinea-pig ileum suspended in Tyrode solution. Bath vol., 70 ml. Upper record, contractions of the longitudinal muscle. Middle record, intraluminal pressure. Lower record, outflow. (*a*) 50 μg. of dimethylphenylpiperazinium (DMPP) added to the fluid bathing the outside of the intestine caused an increase in the activity of the intestinal longitudinal muscle (upper record), but caused inhibition of peristalsis and of flow (middle and lower records). (*b*) 50 μg. of nicotine acid tartrate (N.A.T.) caused inhibition of movement and cessation of peristalsis and of flow.

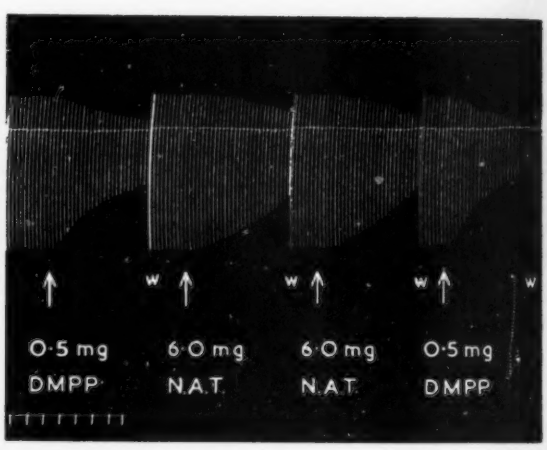


FIG. 6.—Rat phrenic nerve-diaphragm preparation suspended in Tyrode solution. Bath vol., 100 ml. 0.5 mg. of dimethylphenylpiperazinium (DMPP) caused a greater inhibition of stimulation than 6.0 mg. nicotine acid tartrate (N.A.T.). Time, 30 sec.

phenylpiperazinium injected retrogradely into the opposite external iliac artery caused a reduction in the height of the muscle twitch in response to single stimuli applied to the sciatic nerve. The effect produced was similar to that produced by decamethonium (Fig. 7). Much larger doses of tetramethylammonium bromide and of nicotine were needed to produce similar effects. Kaller (1956) suggested that dimethylphenylpiperazinium behaved like decamethonium and suxamethonium, and that it exerted its action by depolarization. This suggestion was supported by the observation that, after the response of the gastrocnemius muscle to stimuli applied to the sciatic nerve had

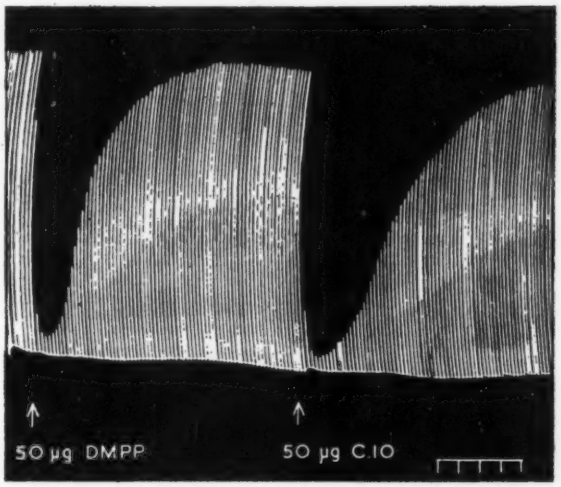


FIG. 7.—Spinal cat. Sciatic nerve gastrocnemius muscle preparation. Injections were made into the opposite iliac artery retrogradely. 50 µg. of dimethylphenylpiperazinium (DMPP) caused a reduction of the response of the muscle to single stimuli applied to the sciatic nerve. The effect was similar to that produced by 50 µg. decamethonium (C.10). Time, 30 sec.

been greatly reduced by dimethylphenylpiperazinium, then during the recovery phase injections of neostigmine produced transient interruption of recovery whereas tubocurarine caused an acceleration of recovery (Fig. 8). In the denervated gastrocnemius muscle preparation, the sciatic nerve having been cut eight days previously, 5 µg. of dimethylphenylpiperazinium given intra-arterially caused a twitch similar in size to, but more sustained than, that produced by 5 µg. of acetylcholine (Fig. 9). 100 µg. of nicotine acid tartrate on the other hand produced a twitch of only half the size.

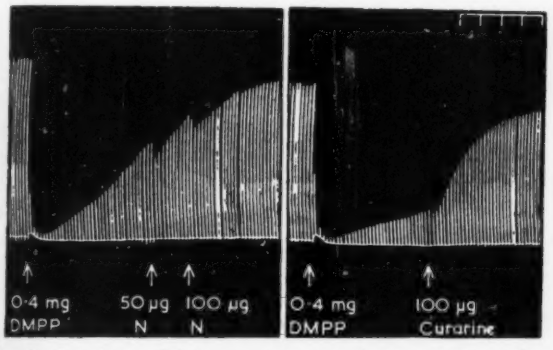


FIG. 8.—Spinal cat. Sciatic nerve gastrocnemius muscle preparation. (*a*) When the response of the gastrocnemius muscle to stimulation of the sciatic nerve was inhibited by 0.4 mg. of dimethylphenylpiperazinium (DMPP), 50 µg. and 100 µg. of neostigmine (N) caused a further transitory inhibition. (*b*) After inhibition caused by 0.4 mg. of dimethylphenylpiperazinium, 100 µg. of tubocurarine chloride (Curarine) caused a return of the response to stimulation. Time, 30 sec.

Effect on Blood Vessels.—Dimethylphenylpiperazinium caused constriction when injected into the vessels of the perfused rabbit ear. Moreover, it usually caused constriction when injected into the hindleg of a dog which was being perfused with blood by means of a Dale-Schuster pump as shown in Fig. 10. There was a rise in systemic pressure and a decrease in outflow. The effect was variable, and in some preparations scarcely any change was seen. In the denervated leg in which the sciatic nerve had been cut 11 days previously, dimethylphenylpiperazinium caused a fall of pressure and an increase in outflow, as it also did in a dog which had received daily injections of reserpine for two days. Under both these conditions nicotine also caused vasodilatation.

DISCUSSION

The stimulant action of dimethylphenylpiperazinium on sympathetic ganglia is well shown in the spinal cat, a preparation which has not been much used by other workers in testing its action. Injection of the drug into the lingual artery (with the external carotid occluded) caused an immediate contraction of the nictitating membrane, the magnitude of the effect being similar to that produced by an equal dose of nicotine. When injected intravenously, however, the drug appeared to be considerably more potent than nicotine: the ratio of the doses of dimethylphenylpiperazinium and nicotine required to produce approximately equal rises of blood

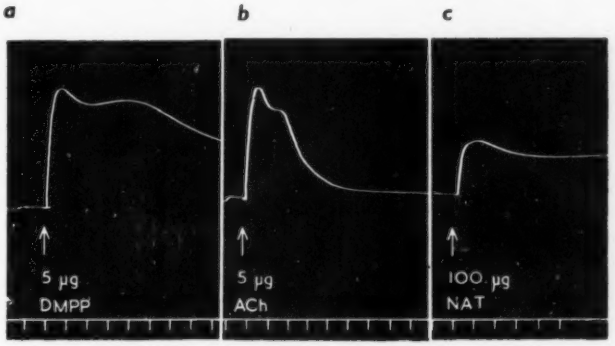


FIG. 9.—Cat, chloralose anaesthesia. Denervated gastrocnemius muscle preparation. Injections were made into the opposite iliac artery retrogradely. (*a*) 5 µg. of dimethylphenylpiperazinium (DMPP) caused a muscle twitch similar to (*b*) 5 µg. of acetylcholine (ACh). (*c*) 100 µg. of nicotine acid tartrate (NAT) produced a smaller effect. Time, 30 sec.

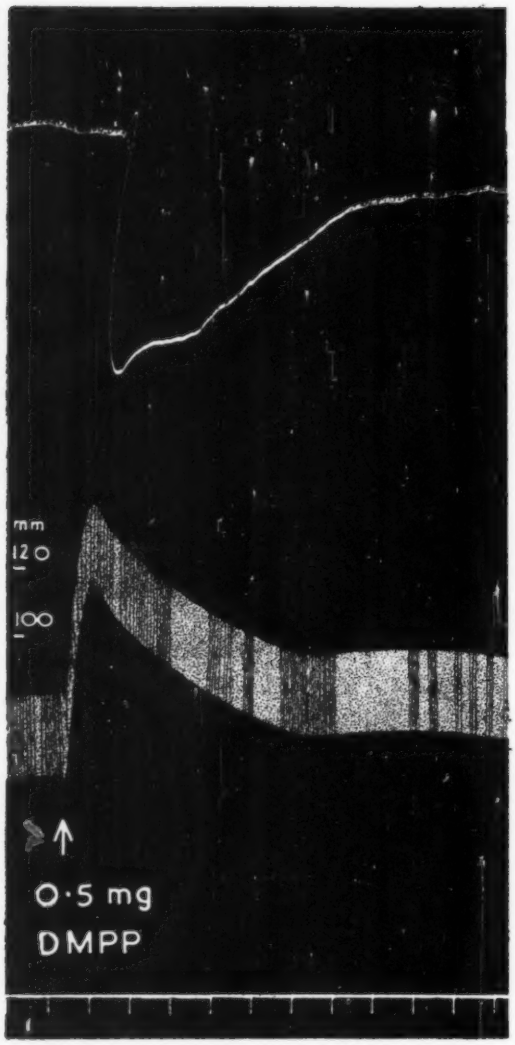


FIG. 10.—Dog hindleg perfused with blood from a Dale-Schuster pump. Upper record, venous outflow. Lower record, arterial pressure. 0.5 mg. dimethylphenylpiperazinium (DMPP) caused a rise in pressure due to vasoconstriction, and fall in outflow. Time, 30 sec.

pressure and contractions of the nictitating membrane was about 1:11 (in terms of base). These two effects were mainly due to the liberation of catecholamines from the adrenal glands, since after exclusion of the glands the pressor effect of previously potent doses was greatly reduced and no contraction of the nictitating membrane was observed. With larger doses of each drug under these conditions, dimethylphenylpiperazinium was about three times as effective as nicotine in causing a rise of blood pressure and contraction of the nictitating membrane.

Recent work by Burn and Rand (1957) has provided new evidence of the release of noradrenaline from skin and blood vessels. Catecholamines, presumably adrenaline and noradrenaline, are known to be present in various organs (Shaw, 1938; Raab, 1943; von Euler, 1946), and noradrenaline was found in the walls of arteries and veins by Schmiterlöw (1948). These observations suggest a possible explanation for the greater potency of dimethylphenylpiperazinium compared with nicotine, when the adrenal glands were excluded: just as dimethylphenylpiperazinium was more potent than nicotine in liberating catecholamines from the adrenal glands, so it may be more effective in releasing them from other tissues. The experiments on the perfused hindleg of the dog support this suggestion: whereas nicotine had very little effect upon this preparation, dimethylphenylpiperazinium always had some constrictor effect and sometimes it was pronounced (see Fig. 10). On the other hand, after previous denervation of the limb or treatment of the dog with reserpine, procedures which deplete the noradrenaline stores, both dimethylphenylpiperazinium and nicotine caused vasodilatation.

Previous workers have shown that dimethylphenylpiperazinium added to the fluid bathing a piece of isolated guinea-pig ileum produced a contraction similar to that produced by nicotine. When the guinea-pig ileum is set up so that peristalsis is recorded, a nervous mechanism is involved with release of the transmitter, and under these conditions nicotine and dimethylpiperazinium caused first stimulation followed by inhibition.

Dimethylphenylpiperazinium had much more effect than nicotine on striated muscle. When injected intra-arterially to the denervated gastrocnemius muscle preparation of the cat it produced a contraction of longer duration but of similar size to that produced by the same dose of acetylcholine. Nicotine produced a much smaller effect in 20 times the dose. The contractions of the normal gastrocnemius muscle preparation produced by stimulation of the sciatic nerve were depressed by dimethylphenylpiperazinium, the inhibition being increased by neostigmine and relieved by tubocurarine. Thus it acted in a manner similar to decamethonium which is known to produce its effect by depolarization.

When dimethylphenylpiperazinium acts alone it has a stimulant action. It stimulated the superior cervical ganglion to cause contraction of the nictitating membrane and the suprarenal

medulla to cause a rise of blood pressure. The guinea-pig ileum was stimulated to cause contraction, as was also the denervated gastrocnemius muscle of the cat when dimethylphenylpiperazinium was injected intra-arterially. When dimethylphenylpiperazinium was given during nerve stimulation, it caused inhibition. Thus it lessened the maintained contraction of the nictitating membrane and reduced the effect of repeated stimuli as was shown by Leach (1957). It inhibited peristalsis produced by raising the intraluminal pressure in the isolated guinea-pig ileum, and it also inhibited the response of the rat diaphragm and the cat gastrocnemius muscle to stimulation through the motor nerve.

Paton and Perry (1953) suggested that the action of nicotine was first to cause a depolarization and then to cause competitive block. It seems probable that dimethylphenylpiperazinium exerts its action by depolarization, causing stimulation of the resting muscle, but causing inhibition by prolonging the depolarization when the muscle is already depolarized. Page and McCubbin (1953) suggested that blocking action was not seen because of the rapid elimination of dimethylphenylpiperazinium from the site of action. This would explain the transitory blocking action, and absence of any subsequent competitive block such as is produced by nicotine.

Because of its good stimulant action and lack of permanent blocking action dimethylphenylpiperazinium would seem to be a useful agent for use in laboratory investigations when repeated stimulation of ganglia is required.

I wish to thank Professor J. H. Burn for giving me the opportunity to do this work and for allowing me to use his preparations of the dog hindleg perfusion. I am also indebted to Dr. E. Bülbring for performing the operation of cutting the sciatic nerve of the cat. My thanks are also due to Messrs. Parke, Davis and Co. Ltd. for a supply of dimethylphenylpiperazinium iodide.

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CONSISTENT DIFFERENCES IN INDIVIDUAL REACTIONS TO DRUGS AND DUMMIES

BY

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The tendency of some individuals to report changes of physical and mental state after taking pharmacologically inert substances has been investigated experimentally. In a class of healthy medical students, those individuals who reported symptoms and those who did not made significantly different scores on a number of behavioural tests. The likely reactions of the members of a second class (containing none of the previous participants) to dummies were then predicted from their scores on the same tests, some of which were found to be much more efficient predictors than would have been expected by chance. Some implications for further research and for clinical medicine are discussed.

Textbooks and pharmacopoeias often imply that sex, age and weight account fully for the variance of human responses to drugs. A contributor to a recent symposium in which the testing of new drugs in man was discussed hoped that " we never come up against differences in human beings. That would make life intolerable." But it has been known for a long time that people differ in their reactions to alcohol (McDougall, 1929) and to salicylates (Hanzlik, 1913) as well as to a large variety of other drugs (see Clark, 1937, for examples) and even to the administration of inert substances (Wolf and Pinsky, 1954, described some particularly striking observations). Beecher (1955) has surveyed a number of earlier reports. However, very little experimental investigation of factors other than sex, age and weight seems to have been undertaken, although it is very probable that the value of clinical trials and the successful treatment of individual patients could both be increased by considering the aspects of the individual's mentality that influence his responses to drugs.

Responses to inert substances were chosen for the present investigation because placebos or dummies (Gaddum, 1954) are frequently used (not always by intention) in clinical and laboratory investigations; because they often have striking effects that, whether useful or undesirable, are not unimportant; and because it is more convenient to give dummies than drugs in the laboratory. The situations in which drugs or inactive substances are given, and the kinds of reaction that may be provoked, have been discussed elsewhere (Joyce, 1959a).

It will be shown that groups of reactors and non-reactors defined by means of their responses to dummies also have characteristically different scores on external criteria unrelated to such responses occurring in the laboratory; that scores on such criteria can predict future responses with more confidence than chance would allow; that the results obtained with healthy students here resemble those obtained in the clinical study of Lasagna, Mosteller, von Felsinger and Beecher (1954); and that the reaction-tendencies of healthy students to dummies resemble those to certain centrally-acting drugs. Future responses were more reliably predicted from responses that had already been made than would have been expected by chance (Wolf, Doering, Clark and Hagans, 1957).

The experiments fell into two phases: In the first, reactor (R) and non-reactor (N) groups were obtained experimentally, and their scores on psychological tests were compared. In the second, the test scores of a fresh group of subjects were used to predict their subsequent experimental responses. A brief account of the first phase has already appeared (Joyce, 1959a): the second phase was reported to the British Psychological Society (Joyce, 1959b).

METHODS

Each phase of the work (Table I) was performed with a completely different class of preclinical medical students, during their normal courses of

TABLE I
EXPERIMENTAL DESIGN

Expt.		Time
Phase One : A	Practical classes : students 1–30 and 31–59	Jan. 1958
B	8 trials of motion-sickness remedies : students 1–59	Jan.–March 1958
C	Psychological and other tests : students 1–59	Jan.–March 1958
Phase Two : D	Psychological and other tests : students 60–118 Prediction of response tendencies	Dec. 1958–Jan. 1959
E	Practical class : students 60–118	Feb. 1959

PRACTICAL CLASS PROTOCOLS

Expt. A		Expt. E	
Time (min.)		Time (min.)	
0–15	Observe symptoms and signs on self and partner	0–20	Symptom questionnaire. Observe symptoms and signs on self only. Pencil and paper tests
15	Drink unknown solution	20	Drink unknown solution
15–60	Continue observing	20–90	Continue observing. Pencil and paper tests
60–75	Symptom questionnaire	90–95	Short symptom questionnaire
75–24 hr.	Continue observing Symptom questionnaire	95	Subjects informed of treatments
		24 hr.	Symptom questionnaire

instruction in pharmacology and psychology. Each class contained 59 members: those in the first were in their third, those in the second in their second preclinical term at the start of the investigation. In the first class 48 were men and 11 women (age range 17.9 to 34.0, mean 21.0); in the second there were 50 men and 9 women (age range 18.1 to 29.1, mean 20.0).

Administration of Inert Substances

Phase One.—Each student was required, as an exercise in making and analysing clinical observations, to record at three-minute intervals respiration rate, pulse rate, and pupillary diameter in himself and a partner for 15 min. before and 60 to 90 min. after ingesting 25 ml. of an unknown liquid. The liquid only contained dilute proprietary orange juice, but it had previously been indicated to the students that the solution might or might not contain an active substance, whose nature (if present) was to be determined by its actions. The result sheets contained space to record additional signs, symptoms, or changes noticed during the experiment. After 60 min. this sheet was exchanged for a questionnaire upon 25 specific symptoms, and this in its turn for a further sheet on which to continue the record for as long as the subject liked. Twenty-four hours later, he completed a further short questionnaire upon long-term symptoms. As the experimental laboratory was small, two such sessions (one for each half-class) were held, one in the morning and one in the afternoon of the same day. The students had been told in the previous week that their reactions were of experimental interest, and that the class thus also involved a piece of serious research in which they were invited to collaborate. The question of volunteering did not arise. The students appeared enthusiastic, and there were only two absentees. The instructions for the practical class were cyclostyled, and the essential minimum of oral explanation was given when individuals asked specific questions. Students remained sitting throughout the class and the instructions discouraged conversation. Each class was supervised by a different demonstrator: that for the first knew that no solution contained an active ingredient, whereas the second did not; but, once the class had begun, neither demonstrator had any contact with the subjects save to collect and exchange their papers.

During the term in which these classes were held (expt. A), Dr. E. M. Glaser examined the side-effects of 4 substances with central actions on the same subjects (expt. B). For 8 weeks, each student measured his pulse rate and answered written questions (Glaser and Whittow, 1954) about 12 specific symptoms during a lecture at the beginning of which (in all save the first week) he had taken a capsule containing either cyclizine hydrochloride (Marzine, 50 mg.), meclozine hydrochloride (Ancolan, 25 mg.), perphenazine (Trilafon, 4 or 8 mg.), prochlorperazine (Stemetil, 13 mg.), or lactose (100 mg.). The drugs are referred to as A to E and F, and the dummy as D. In the first week, no capsules of any kind were given, and in the eighth the treatment of the second week was repeated. There were thus available reports of "symptoms" in the absence of treatment, symptoms following a dummy, and following each of 4 drugs (of which one was given at two doses and one treatment was repeated). In the same term the same subjects took certain simple psychological tests, and personal and academic information was obtained by examinations and further questionnaires. The latter are collectively referred to as expt. C, and are described below.

Phase Two.—The psychological tests previously used were given, with others, at the start of their second preclinical term to a fresh entry of students (expt. D). From their scores on these tests when compared with the results of Phase One, their probable reactions to a dummy in a subsequent practical class (expt. E) were predicted. The form taken by this practical class differed from that in expt. A, because some results from Phase One had been discussed with the participating students about eight months previously. Although at this time the subjects of Phase Two had not yet entered the College, it was considered unsafe to rely upon the division between senior and junior years as an effective barrier to the diffusion of information. Expt. E therefore formed part of a practical class upon the effect of "stimulant" and "depressant" drugs on autonomic responses and simple intellectual tasks. The subjects were told that control substances would also be employed. A single class was held in a laboratory large enough to accommodate all the subjects

comfortably at the same time. They had previously rehearsed the tasks, and now worked independently to a schedule timed for them orally by a single demonstrator. This required respiration and pulse rates and oral temperature to be measured at frequent specified intervals, for 10 min. before and 90 min. after the ingestion of 25 ml. of an unknown solution (see below). The activity of the palmar sweat glands was also estimated with iodine and starch-paper (Randall, 1946). At intervals the subjects carried out letter cancellations, simple additions, and a pencil and paper reaction-time test (Cherry, 1957) arranged for administration to groups. One administration of each kind of test came before and one after the unknown solution was taken. All the material (sheets for recording symptoms, pulse-rates, etc., and those for the tests) was set out at each subject's place before the experiment began; each was turned over and attended to on a signal from the demonstrator. Two spells of 15 min. each were free of experimental measurements, and the students were then allowed to read books that they had brought for this purpose.

The treatments were composed and allocated as follows. The basic medium of *all* solutions was a 1:100 dilution of concentrated compound decoction of aloes (B.P.C.) Of the substances it contains, only aloes itself is recognized to have any pharmacological effect (Goodman and Gilman, 1955), and the lower therapeutic dose to produce catharsis is 120 mg. The solution used here contained 10 mg., and the basic medium was therefore a dummy or more accurately an "impure" placebo. Such a medium was used in order to disguise the presence or absence of active ingredients and to give a characteristically "pharmaceutical" flavour and appearance. It seemed inadvisable to use orange-juice again. Tincture of belladonna was added to some solutions: to some others, caffeine citrate. These substances were used at two concentrations: the upper or average therapeutic doses (respectively of 1 ml. and 250 mg. in 25 ml.) and a 1:1,000 dilution of each (at which dose neither has any known pharmacological effect). The higher concentrations were given to the 9 students who had been absent from the psychological test sessions (expt. D) or whose results had provided insufficient information to predict their reaction to the dummies. The lower concentrations of belladonna or caffeine and the "control" treatment (containing only the medium) were allocated from a table of random numbers to the remaining 50 students. The solutions were drunk simultaneously, within the space of 2 min.: and the vessels were examined afterwards to see if, as was in fact always the case, they had been emptied.

After the observations described had been completed, the record sheets inquired whether each student thought he had taken the "stimulant" or the "depressant," and, if so, on what evidence. After the papers had been handed in the students were told what drugs had been used (but not the concentrations), and to whom each treatment had been given. They did not know the composition of the medium, and no opportunity was given for them to ask questions about the experiment at this point. Twenty-four hours later they completed the questionnaire on long-term symptoms used in expt. A.

Psychological Tests

It was expected from the clinical study already mentioned (Lasagna *et al.*, 1954) that R group members would be more sociable, less "critical," more prone to report symptoms in response to stress in general and more emotionally labile than members of the N group. It was therefore desirable that the information gathered should attempt to measure these characteristics in addition to intelligence and other standard attributes, and the need to carry out these intentions simply, quickly, and simultaneously for the whole group dictated the choice of tests. All the participants were assured that the information to be obtained was for experimental purposes only, and that it would be treated collectively, anonymously, and in the most strict confidence. This assurance was accepted and has not been betrayed: nor does it appear to have been doubted.

The tests that were used follow, roman numerals indicating the phase in which they were given.

(1) "Intelligence": (*a*) Test A.H.5 for administration to highly selected university and professional groups (Heim, 1956) (I, II); (*b*) a modified form of the Critical Thinking Appraisal (Watson and Glaser, 1952) (II).

(2) "Personality": (*a*) Bernreuter Personality Inventory, a test said to measure "dominance" and "sociability" (Flanagan, 1935) (I, II); (*b*) the Maudsley Personality Inventory (the short form of Eysenck, 1958); this estimates "introversion/extraversion" and "neuroticism" (II); (*c*) a modification of the Moreno Sociogram (Moreno, 1953) in which each student rated his relationship with every other in the class on a five-point scale in which the ranks had the following meanings: 0—name unknown; 1—known by sight; 2—occasional social contact; 3—frequent social contact; 4—a friend; 5—a close friend (I, II); (*d*) the Shapes Analysis Test of ability to handle geometric forms mentally (Cane and Horn, 1951) (I); (*e*) Assessment of Autonomic Activity Awareness in which each subject indicated, by marking off on continuous scales (the left ends of which were labelled "Never" and the right ends "Always" but which were otherwise uncalibrated) the extent to which he was aware of his heart action, body temperature, respiration, digestive tract movements, muscular tension and perspiration when in normal health, when anxious, when frightened, and after a pleasurable experience (Mandler, Mandler, and Uviller, 1958).

Information was also collected about performance in objective-test class examinations in pharmacology and statistics (Joyce and Weatherall, 1957) (I, II); "expectation" and judgment of subjects' own performances in class examinations (I, II); rating of preferences for learning by different methods: lectures, reading, and demonstrations, which have

been classified as "passive" methods (Joyce and Weatherall, 1959) and are less "sociable" than practical classes, seminars and tutorless discussion groups, which are also comparatively "active" as far as the student is concerned (I, II). Students also rated their enjoyment of the courses which they had attended, estimated the use each course had been to them, and recorded on individual charts how each working hour during these courses had been spent (I, II). Further information included statements about tobacco and alcohol consumption; willingness to volunteer for experiments of various kinds; self-rating of artistic, linguistic, mathematical, logical, and critical ability. Students in Phase Two were given a questionnaire, one term before experiments D and E, upon their personal use of drugs over the previous year.

Methods of Analysis

In Phase One, the characteristic distributions of the scores obtained on the tests described above by the whole sample and the R and N sub-groups were examined and an appropriate statistical test of the hypothesis that the scores did not differ was selected: this was usually "Student's" t, sometimes preceded by an analysis of variance. For some tests χ^2 or another so-called "distribution-free" statistic (usually the Wald-Wolfowitz runs test [Siegel, 1956]) was appropriate; these will be identified as necessary. Predicted and observed reactions were compared by χ^2 tests, or Fisher's test of exact probability.

Definition of a "Symptom": Method of Scoring Symptoms: Determination of Reaction Types

The rules to be followed were stated after the information in Phase One was collected and before Phase Two began. A symptom was defined as (1) any voluntary written statement about a physical or mental state implying a *change* in function or behaviour in comparison with the control (pre-drug) period; (2) each positive reply in any questionnaire indicating a similar event; (3) the answer "Yes" to either of the specific questions (in expt. C), "Do you think you have taken [either] the depressant [or] the stimulant drug?" One mark was given to any "symptom" so defined. An individual was held to be a reactor on a given occasion if he had a symptom-score of 2 or more or if he answered "Yes" to either question in (3) above. He was considered to be a non-reactor if he had a score of 0 or 1 or answered "No" to *both* questions in (3) above. In Phase One he was called a *consistent* non-reactor if he reported no symptom either in the practical class (expt. A) or on the occasion in the motion-sickness series (expt. B) when the dummy substance was given, a consistent reactor if he reported at least one symptom on each occasion or if his combined symptom-score for the two was 4 or more, and inconsistent if he fell into neither of these groups. In Phase Two the subject's response was considered to be equivocal if the answer "No" to both questions in (3) was accompanied by a symptom-score of 2 or more. Although inconsistent or equivocal responses are of interest, they have been excluded from detailed consideration below.

Results

Phase One

The number of symptoms reported spontaneously in expt. A and in reply to the questionnaires of expt. B formed J-shaped distributions. The latter contained only 12 questions, whereas the questionnaire in expt. A contained 30; and here the responses were fairly symmetrically distributed about a mean of 6 symptoms/subject (Fig. 1).

The total number of attendances in expt. B and of symptoms reported fell slightly, but steadily. Although there was a rather greater tendency for those who had previously made more reports of symptoms to drop out than for those who had

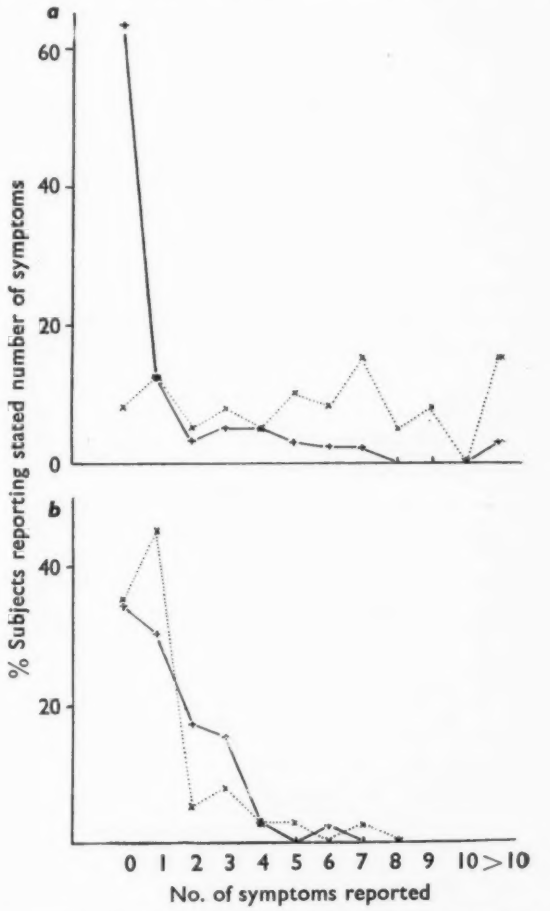

FIG. 1.—Proportion of subjects reporting symptoms. *a*, Expt. A, + —— +, Spontaneous reports (n = 59). X · · · X, Reports to questionnaire on 30 specified symptoms (n = 59). *b*, Expt. B, Reports to questionnaire on 12 specified symptoms, + —— + week 2 (n = 57); X · · · X week 8 (n = 40).

TABLE II

NUMBER OF SYMPTOMS REPORTED FOR EACH TREATMENT IN EXPT. B

Attendances (A) and symptoms reported (S) by whole class (Total), sub-group reacting to dummy (R) and sub-group not reacting to dummy (N); and number of symptoms expected (E) on hypothesis that there are no differences between treatments. D, dummy. For explanation of other treatments see methods.

Treatment:		None	A	B	C	D	E	F	Total	χ^2	P
Total	A	46	50	49	51	54	52	46	348	7·64	>0·2
	S	53	49	59	60	54	53	68	396		
	E	52·3	56·9	55·8	58·0	61·5	59·2	52·3	396		
R	A	24	29	23	28	[31]	28	25	[188] 157	6·10	>0·2
	S	37	28	28	41	[54]	33	40	[261] 207		
	E	31·6	38·2	30·3	36·9	—	36·9	33·0	207		
N	A	22	21	26	23	[23]	24	21	[160] 137	6·37	>0·2
	S	16	21	31	19	[0]	20	28	[135] 135		
	E	21·7	20·7	25·6	22·7	—	23·6	20·7	135		

made fewer reports, this was not significant ($P>0.1$), and there were only small differences in the mean number of symptoms reported for each treatment each week. The distribution of symptoms over treatments for all weeks taken together in the whole group was not significantly different from that to be expected by chance ($P>0.2$), nor were the distributions for the R and N groups taken separately when the responses to the dummy (D) (by means of which the sub-groups were formed) were omitted (Table II).

The mean number of symptoms/occasion reported for the dummy was indeed smaller than that to any drug except A, but the overall differences were insignificant. The number reported after taking drug F was significantly greater than that to the rest combined ($P<0.02$), but as a significance of this order could itself well be attained in one of the set by chance ($P>0.1$) when 6 comparisons are possible (Sakoda, Cohen, and Beall, 1954), the consistency of individual reports about the drug effects was examined on the assumption that the substances did not differ greatly in their pharmacological effects (Table III). The probability that the second reaction could be predicted from the first in any pair of successive occasions more accurately than could be expected by chance was on two occasions highly significant, as was the greater accuracy of the predictions summed over all occasions. The percentage of reactors fell after the start and rose towards the end of the series, but there were no systematic trends in the successful prediction of reactors or non-reactors. Just under two-thirds (63%) of all predictions made were correctly compared with 53% expected to be made by chance. It appeared that, if an interval of a week or longer elapsed between successive attendances, prediction was less successful and, in fact, no better than chance (57% against 56%). However, this sample was too small to justify discussion of the effects upon reaction-tendencies of temporary absence from the experimental situation.

TABLE III

CONSISTENCY OF RESPONSES IN SUCCESSIVE WEEKS

Reactions to drugs or dummy in first week of each pair compared with reactions in succeeding week.

Reactions Observed in Succeeding Week					% Reactions Correctly Predicted in Succeeding Week				
Reactions Observed in First Week of Each Pair		R	N	% R	R	N	Total	Chance	P
1	R	22	8	64	69	56	64	53	>0·1
	N	10	10						
2	R	23	11	63	68	45	59	53	>0·5
	N	11	9						
3	R	18	7	56	75	63	70	51	<0·02
	N	6	12						
4	R	9	9	42	56	59	58	51	>0·5
	N	7	13						
5	R	7	6	37	54	73	66	53	>0·2
	N	6	16						
6	R	12	2	54	63	88	74	49	=0·0003
	N	7	14						
7	R	12	6	65	55	50	53	51	>0·8
	N	10	6						
(Total all pairs)	R	81	41	54	63	65	63	53	<0·001
	N	47	70						
Before absence	R	14	8	63	71	27	57	56	=0·765
	N	5	3						

Scores on Psychological and Other Tests

In all, the scores of the R and N sub-groups were compared on a total of 30 measures derived from the tests described above (including some, such as age and intelligence, that were not expected to discriminate between the groups). In a set of this size, about 3 results significant at or better than the 10% level would be expected by chance, of which 1 or 2 would also be significant at or better than 5%, but none at or better than 1%. The number of tests actually observed to reach each level was 12, 6 and 2 respectively

(Table IV: all the results attaining the 10% level or better are tabulated with others of interest). The likelihood of obtaining a constellation of scores such as this by chance is less than one in a thousand (Sakoda *et al.*, 1954).

The Bernreuter Inventory scores showed the R group to be insignificantly more "extraverted" and "sociable" and significantly less "self-confident" and "dominant" in personal relationships than the N group. These measures are known to be highly intercorrelated (Flanagan, 1935) and only the scores on the orthogonal factors of "self-confidence" and "sociability" have been considered here. The smaller degree of self-confidence of the R group was supported by the difference in judgments of their own performance by members of the two groups in the class examination, which was of borderline significance. The R group judged their own performance more severely, and indeed underestimated it: the N group overestimated theirs. There was no difference between the groups, however, on judgments about performances not related to class-work.

The greater "sociability" of the R group was also supported in several other ways: first, the sociograms showed that the R group gave a higher average "vote" to their class colleagues, and were also rated more highly by them; second, the R group indicated before the course began a greater preference for methods of teaching which depended upon group activity; and third, they showed after the course was over that they had valued such methods more highly. Finally, the other scores were taken to indicate that the R group were "less critical" of the teaching that they had received, in that the scores they awarded for their enjoyment of the course as a whole and for their view of the use it had been to them agreed to a significantly greater extent than did those of the N group, whose answers suggested either that they had enjoyed the course more than they had benefited from it, or the contrary, and so perhaps showed that they thought it deficient in one respect or another. The significant tendency of the groups to seek different amounts of further teaching may perhaps also indicate that the N group thought the course of less use. The number of "symptoms" reported in expt. B when no substance, not even the dummy, had been taken, the total number of occasions on which symptoms were reported, and the average number of symptoms/attendance were all greater for the R than for the N group, but the differences were of doubtful significance. There were no differences in the respiration rates or pupillary diameter of

TABLE IV

SCORES OF CONSISTENT REACTORS (R) AND NON-REACTORS (N) ON PERFORMANCE TESTS

All probabilities based on 2-tailed t-test except for those indicated by asterisk (χ^2 or Wald-Wolfowitz runs test): probabilities >0·1 shown to nearest lower 0·1: "X" in Phase Two indicates difference in non-predicted direction.

	Phase One				Phase Two			
	R	N	S.E. $(\bar{R}-\bar{N})$	*P*	R	N	S.E. $(\bar{R}-\bar{N})$	*P*
n: total	10–13	11–15	—	—	18	13	—	—
Men	11	13	—	—	11	13	—	—
Women	2	2	—	—	7	0	—	—
Age	20·1	20·6	0·58	>0·3	19·9	20·4	0·52	>0·3
A.H.5: intelligence	33·2	33·0	3·60	>0·9	32·1	34·8	4·15	>0·5
F.1: self-confidence	+6·7	−55·5	20·74	<0·01	−1·1	−28·7	30·10	>0·3
F.2: sociability	−22·8	−11·7	23·71	>0·6	−44·6	−30·1	20·54	>0·4
Sociogram: of others	128·8	120·0	13·25	>0·5	137·0	136·0	14·60	>0·9
Sociogram: by others	125·0	111·4	7·81	0·1>*P*>0·05	139·5	147·5	14·86	X
Shapes Analysis	17·6	18·4	1·94	>0·6	—	—	—	—
Class examination	56·0	57·7	4·78	>0·7	—	—	—	—
Judgment of performance	49·9	62·9	7·25	0·1>*P*>0·05	—	—	—	—
Private study/week (hr.)	8·2	8·4	2·26	>0·9	13·8	13·9	1·75	>0·9
Course use/enjoyment	1·0	0·8	—	<0·05*	1·4	0·8	—	0·1>*P*>0·05*
Requests for more teaching	3·6	3·8	—	<0·05*	—	—	—	—
Group methods preference:								
Before course	5·2	3·6	—	0·05>*P*>0·02*	6·3	4·3	—	>0·3*
After ,,	5·3	3·6	—	0·05>*P*>0·02*	—	—	—	—
Willingness to volunteer	5·7	6·3	0·83	>0·4	5·5	5·5	0·54	>0·9
Resting "symptoms" (no drug)	1·4	0·7	0·45	>0·1	4·8	4·1	0·80	>0·3
Occasions with symptoms	4·4	2·9	0·84	0·1>*P*>0·05	—	—	—	—
Symptoms/attendance	1·4	0·9	0·36	>0·2	—	—	—	—
Resting pulse	76·1	71·5	1·47	<0·01	82·5	81·2	2·92	>0·6
Resting pulse: coefficient of variation	9·9	6·6	1·70	0·1>*P*>0·05	7·7	9·2	1·22	X
% change after "drug"	7·7	5·0	1·38	0·1>*P*>0·05	4·3	7·1	2·00	X

the groups: the resting pulse rate of the R group was significantly higher, however, and showed greater variation: the fall in pulse rate immediately after taking the "drug" was also greater. The last two effects were of borderline significance.

No attempt was made to subject the results to factor analysis, because the tests were "notional" or at most empirical (Eysenck, 1959) rather than founded on theory. But real differences were clearly detected on occasions that were quite distinct from those in which the criterion reaction (to the dummy itself) was demonstrated, and it was this and the prognostic use of such tests that was important rather than the attribution of a label to what was being measured.

In Phase Two the tests were used prognostically. The psychological information about a second set of students was collected first, and from this the probable reaction of each to the administration of an inert substance on a subsequent occasion was predicted. The results of Phase One suggested that information from students whose native language was not English was not strictly comparable with that from their fellows, and such subjects were excluded *a priori* from consideration in Phase Two, although they sat the tests and attended the practical class.

The Bernreuter responses of the R and N groups in Phase One were subjected to an item analysis, and twelve questions to which the responses of the two groups were found to be characteristically different ($P<0.1$) were extracted. One mark was given for each response typical of the N group: and the scores of the fresh groups reacting and failing to react to the dummy in expt. E were significantly different. Scores on this set of questions (the "RN scale") were also used to predict, in advance of expt. E, whether each individual would or would not be a reactor to the dummy. The outcome (Table V) was highly successful, and may be compared with the predictions based upon previous individual responses in expt. B (Table III). No other single predictor (pulse rate, self-confidence, etc.) had better than 60% success, which was not significantly above that to be expected by chance; and no combination of three predictors in a discriminant was found that was as good as the RN scale alone.

TABLE V

PREDICTION OF REACTIONS IN EXPT. E FROM SCORES ON RN SCALE

Probability of this distribution (Fisher's exact test): <0.0002.

		Observed Reaction-type	
		R	N
Prediction: RN scale	R	16	4
	N	2	9

The right-hand part of Table IV gives the scores in Phase Two on those tests that were common to the two phases, and indicates whether or not the trend of the differences seen in Phase One was confirmed. This was the case in all save one of the psychological tests that were significant in Phase One, although the differences in Phase Two did not reach conventionally acceptable levels of significance for individual tests. But the probability of obtaining a set of results showing a consistent trend of this magnitude by chance is very small ($P<0.005$); and even if the results from the three tests upon the pulse rate (two out of three of which were in the unexpected direction) are included, the overall probability is still such that the set of observations is very unlikely to have arisen by chance ($P<0.03$).

Other tests were given for the first time in Phase Two (Table VI). Those who reacted to the dummy scored higher on the "Extraversion" and "Neuroticism" indices of the short Maudsley questionnaire, but not significantly so: and significantly higher on the autonomic questionnaire, thus admitting to a greater awareness (or willingness to report awareness) of such bodily events as bowel movements, sweating and so on, both at rest and under various kinds of stimulation, than did the N group. The groups did not recall significantly different numbers of occasions on which they had made use of drugs during the previous year, nor did they report different

TABLE VI

SCORES OF R AND N GROUPS IN QUESTIONNAIRES USED FOR THE FIRST TIME IN PHASE TWO

All probabilities based on 2-tailed t-tests: probabilities >0·1 shown to nearest lower 0·1. MPI, Maudsley Personality Inventory. BPI, Bernreuter Personality Inventory.

Test	R	N	S.E. $(\bar{R}-\bar{N})$	P
No. of participants	18	13		
MPI (short form)				
Neuroticism	+1·28	−0·08	1·205	>0·2
Extraversion ..	+0·52	−0·15	1·125	>0·5
Autonomic Awareness Questionnaire	90·9	69·0	10·22	$0·05>P>0·02$
BPI "no" answers ..	55·1	61·6	3·67	$0·1>P>0·05$
RN scale ..	6·8	8·8	0·97	$0·05>P>0·02$
Experience with drugs:				
No. taken in previous year	2·1	2·3	0·46	>0·6
Symptoms experienced/drug ..	2·5	2·4	0·42	>0·9
Symptoms expected ..	4·3	3·5	0·97	>0·4

recollections of the drug effects. There was a suggestion that the R group "expected" to experience more side-effects from any future administration of a drug than did the N group.

Save for the Autonomic Awareness test, which consists of interval-free scales that require no verbal response, the questionnaires force a Yes/No or "Don't know" choice to be made for each question. It seemed possible that the R group might be less willing to answer with a categorical "No," and this was found to be true for the Bernreuter Inventory as a whole.

Discussion

The reactions to inert substances of healthy young adult subjects were clearly much more consistent and predictable than would have been expected by chance, whether previous reactions or suitable independent pencil and paper tests were used to make the prediction. This cannot have been due to the fact that the critical experiments were not all "double-blind." It is true and it was inevitable that an experimenter sometimes knew which subjects were receiving the dummy, because these were always in the majority and sometimes the entire group. But the purpose of the critical experiments was to divide the group into those who reacted and those who did not, and the experimenter had no advance information to suggest which subjects would be which in expts. A or B (in the latter of which active substances were also used in a double-blind design) and he was unaware of the *predicted* responses in expt. E, for these had been worked out in coded form. The success of the methods used to predict responses might well have been even greater had the experiments in the two phases resembled each other more closely. The reasons for deliberately varying them have already been discussed and attention has been drawn to the influence of the length and form of questionnaires upon the distribution of the responses, as well as that of whether questionnaires are used at all. The degree of success achieved is therefore satisfactory.

The experiments do not show whether reactions to drugs resemble those to dummies: the very small difference in the number of reactions to the dummy and those to the active agents in expt. B (Table II) suggested that the latter, with the possible exception of drug F, were indistinguishable from dummies, and the fact that these reactions could be predicted as accurately (although by different means) as those in expt. E is therefore not strong evidence on this point. On the other hand the resemblance of such psychological information as has been associated with the specific reaction tendencies of these students to that obtained by Lasagna and his colleagues (1954) from their surgical patients suggests that the clinical and experimental situations can be validly compared. Again, the clinical information was obtained in very different ways (psychiatric interviews, evaluation by nursing staff and Rorschach testing) from those used here (objective measures) and the success of the comparison is therefore encouraging. However, it is as easy and dangerous in the field of "personality" studies to multiply entities as to call different entities by the same name, and a study is now in progress in which the measures derived from healthy subjects are being applied to patients.

No attempt has been made to find "labels" for the differences that have emerged by applying factor analysis without a basis of previous theory: but the results strongly suggest that R and N reactions or reactors constitute the extremes of a continuous distribution with the inconsistent reactors occupying a middle position, for the tests that distinguish significantly between the two groups always, and the remainder frequently, give group mean scores that lie on opposite sides of the mean for the whole group of 59 subjects. Three tests in particular are efficient discriminators: the RN scale, the Autonomic Awareness questionnaire, and the actual response to a dummy: with their aid it seems likely that the factors in the subject and in his environment that combine to produce in him a tendency to react or fail to react can be investigated in the future. The identification of these factors is likely to be worth while for the reasons discussed below, and it is encouraging that they can be studied relevantly in the experimental laboratory.

First, in the clinical evaluation of new remedies. Well-designed clinical trials compare new treatments with the best currently available or, if none yet exists, with a regime that is similar in all respects to the experimental, save that the agent under investigation is omitted. If the treatment investigated is actually effective, a high proportion of reactors (in relation to that present in the population at large) in the experimental groups may tend to decrease the apparent value of the drug. Too many non-reactors in the experimental sample, on the other hand, will tend to cause disappointment when the new drug is brought into general use, because the difference between the results of treatment with the active and control substances has been accentuated in the trial. Self-controlled trials are not always feasible:

and although wherever possible this design should be adopted it will not in fact eliminate the difficulty under discussion. The alternative use of matched groups for treatment and control will not be satisfactory if it does not include a simple test of reaction tendency: indeed, whichever design is used it is probably necessary to show that the proportion of reactors and non-reactors in the group (or groups) resembles that in the general population. It has sometimes been proposed that reactors or even non-reactors should be excluded from clinical trials, but the consequences of this would appear to be even more misleading: though even if this were not so the need to test reaction-tendencies would not be avoided.

Second, in deciding upon the correct treatment for the individual patient. There are some circumstances in which the withholding of a potentially dangerous drug, provided that equal benefit can be obtained from an inert or harmless treatment, is not merely justifiable but probably correct. The truth of this proposition must be examined separately for each patient, but the condition from which the patient to be tried on placebo is suffering should be non-terminal and preferably spontaneously remitting, speedily improved by treatment with the active substance should the placebo fail, and not adversely affected by initial failure to give stronger medication.

The consequences of initially assigning the individual patient to the wrong category must be considered. He may in fact be a reactor, although classified as a non-reactor, or the contrary. In the first case he will be unnecessarily treated with the active drug, in the second incorrectly with the placebo, and the implications of the errors are different. The first course will expose him to the risk of side-effects, addiction, etc.: the latter may cause him to suffer slightly longer than would have been the case had he been correctly placed. No biological predictive instrument can be expected to have an efficiency that approaches 100%, so errors of both kinds will inevitably be made. But such errors are continually and inevitably being made therapeutically, and the use of a rational instrument of the kind outlined here should in some circumstances enable them to be reduced. In fact, errors of misclassification in the experiments reported above amounted to about 37% (Table III) and 19% (Table IV): in both cases, each kind of error contributed about 50% of the total. These errors should certainly be further reduced when the predictive technique is refined.

Third, in investigating adjuncts to other forms of therapy. The reaction to an active drug depends upon the drug itself, the patient's history, his present state, his doctor and other ministrants (Feldman, 1956; Sabshin and Ramot, 1956; Uhlenhuth, Canter, Neustadt and Payson, 1959). The effect of even active drugs can be increased by giving them in the most suitable circumstances, and information about the psychic factors in the patient predisposing him to react favourably is therefore as important to the doctor as knowledge of the patient's body temperature, childhood illnesses and attitude to his job. Such factors may perhaps not be more difficult to estimate reliably than the latter.

Finally, as far as human pharmacology is concerned, this kind of study may illuminate some causes of variation in controlled treatments that are usually ignored or accepted with resignation. The main emphasis in psychopharmacology until now has been on drugs rather than souls: less interest has so far been shown (with notable exceptions such as Eysenck (1957)) in using drugs to display and estimate differences between healthy nervous systems, but the occurrence of reactions to inactive substances is important to studies of both kinds.

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THE CENTRAL ACTION OF LIGNOCAINE AND ITS EFFECT ON CARDIAC OUTPUT

BY

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Fifty-nine tests were performed on twenty-four anaesthetized dogs in a study of the effects of lignocaine on circulatory and respiratory functions. In single-dog experiments, it was shown that lignocaine (1 to 2 mg./kg. body weight) increased ventilation somewhat, but had no effect on O_2 consumption. Cardiac output was increased due to a rise in both heart rate and stroke volume. There was an elevation of arterial blood pressure. The central blood volume was increased, but central venous pressure, total peripheral resistance and the ventilation-perfusion ratio were decreased. The increase in cardiac output after lignocaine was abolished both in decerebrate and in vagotomized dogs. In cross-circulation experiments with dogs, it was demonstrated that the primary site of action of lignocaine on cardiac output was central. The effect of lignocaine on cardiac output could be blocked by intravenous procaine.

Lignocaine increases the arterial blood pressure in man (Kimmey and Steinhaus, 1959) and in dogs (Carden and Steinhaus, 1956) when the dose is limited to 1 to 2 mg./kg. body weight. This effect differs from that of procaine, which decreases arterial blood pressure (Edmonds, Comer, Kennedy, and Taylor, 1949). How lignocaine produces this rise in blood pressure has not been explained. Possible mechanisms include: (1) a peripheral action on the heart or the blood vessels or both; and (2) a direct action on the brain, spinal cord or other elements of the nervous system. It was thought that these possible mechanisms might be analysed in decerebration experiments (in which central effects might be abolished) and in cross-circulation experiments (with central and peripheral effects isolated physiologically).

This paper is a report of an investigation of these possibilities. Special attention was paid to changes in cardiac output due to lignocaine.

METHODS

Fifty-nine experiments were performed on twenty-four mongrel dogs of both sexes, weighing 9 to 21 kg. and anaesthetized with either pentobarbitone sodium (30 mg./kg., body weight, intravenously) or a chloralose and urethane mixture (50 and 500 mg./kg. respectively). In decerebration experiments the midbrain was transected (Kao, Schlig, and Brooks, 1955) under thiopentone sodium (25 mg./kg.) anaesthesia.

For cross-circulation experiments, dogs of similar weight were selected. The head of the recipient dog was perfused exclusively by blood from a second dog, the donor. This preparation is depicted in Fig. 1. The technique and its validity have been discussed previously (Kao, 1956; Harmel, King, and Kao, 1958). In brief, the head of the recipient was supplied through its carotid arteries by blood (from the donor's common carotids) which was drained back to the donor by external jugular veins of the recipient. The vertebral arteries of the donor dog supplied its own head, but the vertebral arteries of the recipient dog were tied.

A tracheal cannula was inserted in each dog and connected to a Benedict-Roth spirometer which was filled with pure O_2 and arranged for continuous recording of tidal volume, respiratory rate and O_2 consumption.

In single-dog experiments, a catheter (internal diameter 0.085 in.; external diameter 0.128 in.) was inserted into one of the common carotid arteries and arterial blood pressure measured by means of a mercury manometer or a Statham strain-gauge. Pressure changes were monitored by a Sanborn Recorder. Usually in cross-circulation experiments the femoral arterial blood pressure of the donor dog was registered with a mercury manometer while femoral arterial blood pressure of the recipient dog was recorded by means of a transducer and the Sanborn Recorder. Heart rate was monitored continuously by a Lead I electrocardiogram.

Cardiac output was determined by the dye-dilution technique. Cardiogreen (2.13 mg.) was delivered through a BD 442T catheter to the right atrium and the dye concentration in the arterial blood was

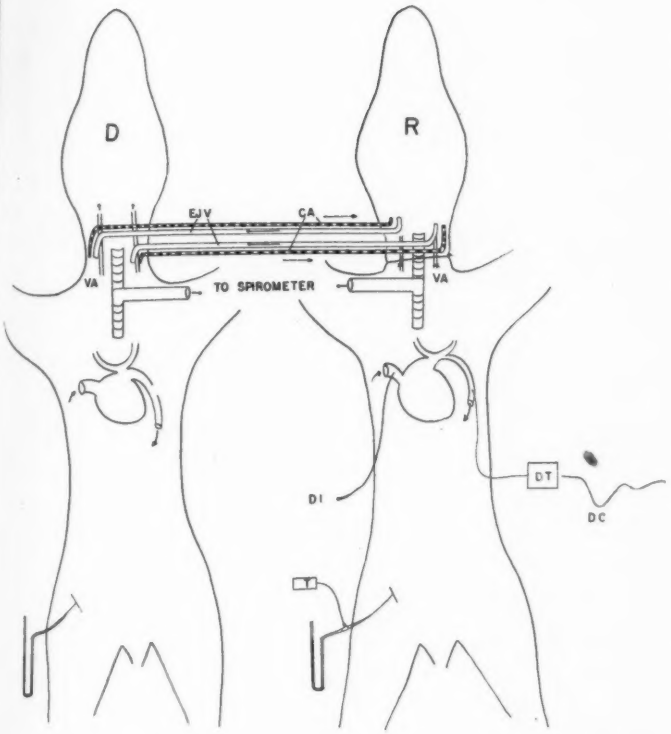

FIG. 1.—Diagram of the arrangement for cross-circulation experiments. **D**, donor dog; **R**, recipient dog. **VA**, vertebral artery; **EJV**, external jugular vein; **CA**, carotid artery; **DI**, site of dye injection; **DT**, densitometer; **DC**, dye curve; →, direction of blood flow.

continuously monitored through a Colson densitometer and recorded on a Sanborn Recorder. A catheter (internal diameter 0.106 in.; external diameter 0.138 in.) was introduced into the femoral artery and passed into the aortic arch for sampling the arterial dye concentration. In cross-circulation experiments only the cardiac output of the recipient dog was measured.

Additional experiments were performed in dogs with bilateral cervical vagotomy and in dogs after the administration of procaine (1 to 2 mg./kg.) to determine the effect of procaine alone and its blocking action on lignocaine.

Central blood volume was calculated by multiplying the circulation time in sec. and the cardiac output in ml./sec. The volume thus obtained is actually the blood volume by which the dye is diluted between the site of dye delivery and the site of dye sampling. This volume is somewhat greater than the true pulmonary blood volume. A change in central blood volume, however, reflects that of the pulmonary circulation, since any change in heart chamber volume is very small in comparison with the volume of the pulmonary vascular bed and since the amount of blood in the aortic arch can undergo no significant change.

The total peripheral resistance was calculated by employing the following conventional formula: total peripheral resistance = (arterial blood pressure − central venous pressure) × 1,332/cardiac output. Pressures were expressed in mm. Hg, and cardiac output in ml./sec. Total peripheral resistance was expressed in dyne-sec./cm.[5]

Biometrical analyses were as described by Snedecor (1946). All drugs were injected intravenously unless otherwise stated.

RESULTS

Effect of Lignocaine on Ventilation and O_2 Consumption

Since a change in O_2 consumption affects cardiac output (Kao and Ray, 1954) it seemed necessary to determine whether lignocaine increased O_2 consumption. O_2 consumption and ventilatory values before and after injection of lignocaine (1 mg./kg.) are shown in Table IA. The average increase in O_2 consumption (2.8 ml./min.) following lignocaine was not significant statistically; the total ventilation, however, increased from an average of 2.991 to 3.419 l./min. (difference, 0.422 l./min., $P<0.01$) though the change lasted only a few minutes. Maximal values were used for this part of the analysis.

Effect of Lignocaine on Cardiac Output and Peripheral Circulation

Cardiac output increased in the first minute after the injection of lignocaine. The maximal effect was attained within 10 min. and the increment lasted 40 min. Arterial blood pressure followed the same general course as the cardiac output, rising to a peak value during the first 10 min. and then declining gradually in the subsequent 30 min.

The various circulatory effects of lignocaine are shown in Table IB. In brief, lignocaine caused an increase in cardiac output by increasing both heart rate and stroke volume, but produced no change in O_2 consumption; thus the circulatory equivalent for O_2 (Kao and Ray, 1954) increased. This signifies that for each unit of O_2 demand made by body tissues, blood flow was available to supply this O_2. Arterial blood pressure was elevated by 11.3 mm. Hg, while the central venous pressure was not decreased significantly. The total peripheral resistance also decreased, which implied that there was a peripheral vasodilatation.

TABLE I

ACTIONS OF LIGNOCAINE (1·0 MG./KG., INTRAVENOUSLY)

The results are means of eight control and lignocaine-treated dogs (n=8). O_2 consumption is expressed at standard temperature and pressure, dry; tidal volume and total ventilation are expressed at body temperature and pressure, saturated.

Variables	Control	Lignocaine-treated	Mean Difference	t	P
A. O_2 consumption and ventilatory values					
O_2 consumption (ml./min.) ..	97·1	99·9	2·80	1·5873	<0·15
Respiration rate (breaths/min.) ..	15·5	18·4	2·9	1·0324	>0·30
Tidal vol. (ml.) ..	230·3	244·8	14·5	1·0469	>0·30
Total vent. (l./min.)	2·991	3·419	0·422	2·8691	<0·01
B. Circulatory responses					
Cardiac output (l./min.) ..	2·514	3·415	0·901	6·8854	<0.01
Heart rate (beats/min.) ..	139·5	158·1	18·6	3·2871	<0·01
Stroke vol. (ml.) ..	18·775	21·938	3·163	3·4117	<0·01
Arterial blood pressure (mm.Hg)	134·5	145·8	11·3	5·2055	<0·01
Central venous pressure ($cm.H_2O$)	4·88	4·63	−0·25	0·1517	>0·50
Total peripheral resistance (dyne-sec./$cm.^5$)	4,368·8	3,442·5	−926·3	3·7651	<0·01
Central blood volume (ml.) ..	308·0	397·4	89·4	5·6312	<0·01
Ventilation-perfusion ratio	1·206	0·996	−0·210	4·2955	<0·01

It is interesting also to note that the central blood volume increased significantly, which means that a dilatation of the pulmonary vessels occurred, a process that is advantageous to the gas exchange in the lungs. Since the ventilation increased little compared to cardiac output, the ventilation-perfusion ratio (ventilation in l./min./cardiac output in l./min.) decreased.

Fig. 2 illustrates quantitatively the effect of lignocaine on cardiac output. The maximal response was obtained when the dose of lignocaine was about 1.5 mg./kg. Above this dosage, the rise in cardiac output was definitely smaller.

Lignocaine in Vagotomized Dogs

Eight experiments were performed on vagotomized dogs. Following bilateral cervical vagotomy, cardiac output increased in association with an elevation of arterial blood pressure to 145 to 180 mm. Hg and a rise in heart rate to 125 to 195 beats/min. When very small doses of lignocaine were given there was little further increment in cardiac output, and when the lignocaine dosage was increased cardiac output actually decreased (Fig. 3). This fall was due to a decrease in stroke volume since heart rate was increased significantly following all doses of lignocaine. Arterial blood pressure and the total peripheral resistance also decreased below the post-vagotomy level after large doses of lignocaine. Such falls did not follow similar doses of lignocaine before the vagi were cut.

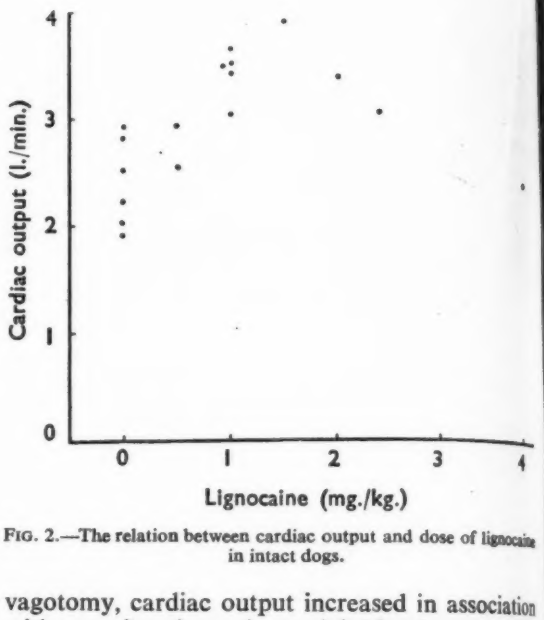

FIG. 2.—The relation between cardiac output and dose of lignocaine in intact dogs.

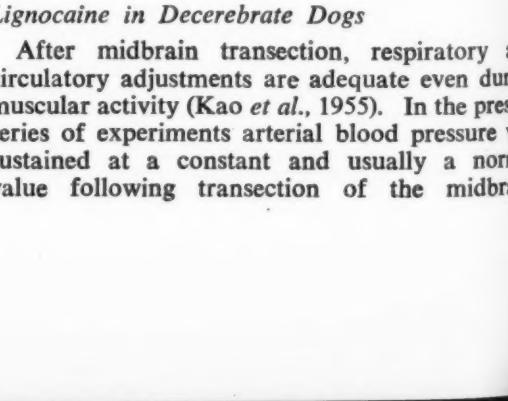

FIG. 3.—The effect of vagotomy on the changes in cardiac output due to lignocaine. Cardiac output is expressed as the ratio of values before and after various doses of lignocaine.

Lignocaine in Decerebrate Dogs

After midbrain transection, respiratory and circulatory adjustments are adequate even during muscular activity (Kao *et al.*, 1955). In the present series of experiments arterial blood pressure was sustained at a constant and usually a normal value following transection of the midbrain.

When lignocaine was given to these dogs even in small doses cardiac output was invariably decreased (Fig. 4). The fact that after midbrain transection and vagotomy lignocaine did not augment cardiac output suggests a central action of the drug. It was thought, however, that the drastic procedures employed might have masked any reaction. Therefore the cross-circulation technique was employed to test the hypothetical central action of the drug.

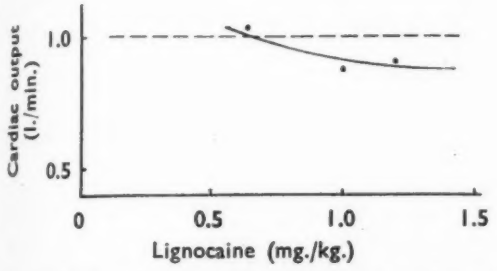

FIG. 4.—The effect of decerebration on changes in cardiac output due to lignocaine. Ordinate and abscissa as in Fig. 3.

Lignocaine in Cross-circulation Experiments

Twenty tests were carried out in four pairs of dogs with cross-circulations. When lignocaine was injected into the body of the recipient dog there was no change in cardiac output of the recipient dog (Fig. 5). This made it quite clear that lignocaine had no peripheral effect on the circulation. It is important to note that this finding constitutes further evidence that there was no appreciable blood supply to the head of the recipient dog from its own body. When lignocaine was injected into the blood supplying the recipient's head, the cardiac output of the recipient dog did increase. This effect was either markedly diminished or abolished after bilateral vagotomy in the recipient dog (Fig. 5).

Lignocaine in Procainized Dogs

Twenty tests were performed on six dogs into which various doses of procaine (0.3 to 2.0 mg./kg.) had first been injected. These doses of procaine had no observable effect on cardiac output or the peripheral circulation. But, when lignocaine (1 mg./kg.) was given to these dogs after treatment with procaine, the cardiac responses varied as a function of the amount of procaine employed. This is shown in Fig. 6. It can be seen that, when the procaine dose reached 1 mg./kg., the effect of lignocaine on cardiac output was abolished. After smaller doses of procaine lignocaine still caused some rise in cardiac output.

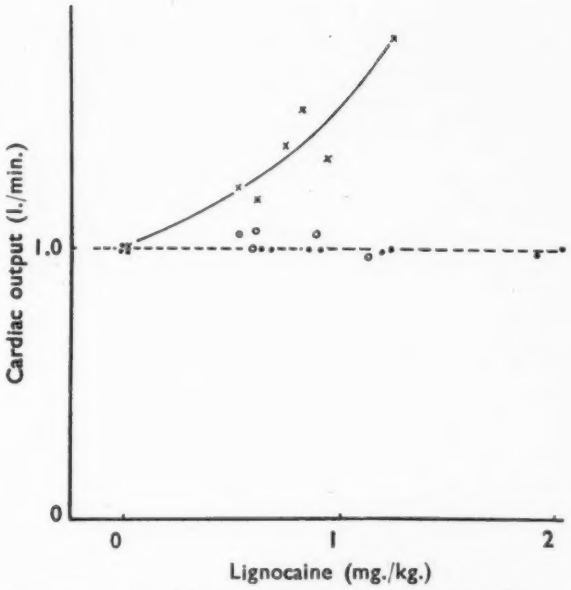

FIG. 5.—Cross-circulation experiments. The effects of lignocaine on the cardiac output of the recipient dog (expressed as in Fig. 3). Lignocaine was injected into the circulation of the recipient's head with vagi intact (X) and after vagotomy (O). There was no effect when lignocaine was injected into the recipient's body (●).

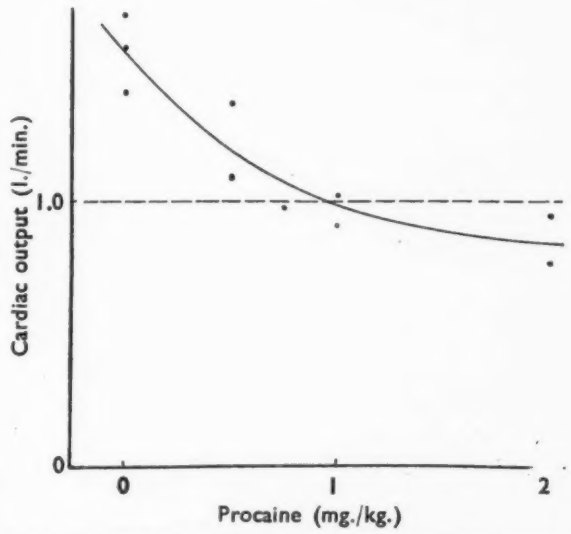

FIG. 6.—Effects of graded doses of procaine on the action of lignocaine (1 mg./kg.) on cardiac output (as expressed in Fig. 3).

DISCUSSION

Lignocaine possesses a variety of actions in mammals including man. Early studies were focused mainly on its local anaesthetic effect (Goldberg, 1949; Gordh, 1948; Wiedling, 1952). Kimmey and Steinhaus (1959) demonstrated that lignocaine raised the arterial blood pressure when

the intravenous dose was limited to 1 to 2 mg./kg. The mechanism of this effect was not explained. From the present study it seems clear that lignocaine has multiple effects on the circulation, and the elevation of arterial blood pressure is but one of them.

Lignocaine caused a peripheral vasodilatation, but the central venous pressure did not increase. This suggests that the increase in arterial blood pressure is due primarily to an increased contractile force of the heart or to an increased cardiac output not related to the peripheral vasodilatation. Our results show that lignocaine increases cardiac output by acting not on the heart directly but on the brain.

The exact site of this action in the brain has yet to be determined. It is evident, however, that both sympathetic and vagal pathways are involved. The rise in cardiac output is due to an increase both in stroke volume and heart rate. The increase in heart rate is probably due to sympathetic stimulation and the increment in stroke volume does not occur after vagotomy. It seems justifiable to state that lignocaine acts on autonomic areas in the central nervous system.

In view of the fact that lignocaine provokes a peripheral vasodilatation, a pulmonary vasodilatation and an increase in cardiac output, gas exchange in the body is enhanced. It may, therefore, be inferred that lignocaine might be useful in dealing with certain cardiac and circulatory disturbances.

The authors wish to express their gratitude to Dr. A. P. Truant for his invaluable suggestions and ideas in the present investigation. Lignocaine (Xylocaine) was kindly supplied by Dr. A. P. Truant, of Astra Pharmaceutical Products. This investigation was supported in part by grants from the National Institutes of Health, U.S. Public Health Service (B-1907), and from Astra Pharmaceutical Products, Inc., Worcester, Mass., U.S.A.

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PHARMACOLOGICAL PROPERTIES OF PHENYLDIGUANIDE AND OTHER AMIDINE DERIVATIVES IN RELATION TO THOSE OF 5-HYDROXYTRYPTAMINE

BY

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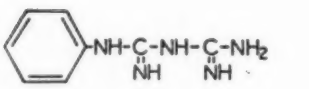
Cats in which the coronary and allied chemoreflexes could not be obtained with small intravenous doses of 5-hydroxytryptamine were insensitive also to phenyldiguanide. In cats which responded to phenyldiguanide with reflex falls of blood pressure and heart rate, abolished by vagotomy, the effects of graded doses (5 to 150 μg./kg.) of phenyldiguanide bore a striking resemblance to those produced initially by 5-hydroxytryptamine in somewhat smaller doses. Differences in the cardiovascular responses to the two drugs are attributed to additional (non-reflex) actions of 5-hydroxytryptamine. The reflex actions of both drugs were blocked reversibly also by 2-naphthylguanidine (500 μg.). Certain other drugs (bufotenine, procaine, *S*-decylisothiourea) antagonized the depressor action of phenyldiguanide as well as the reflex depressor action of 5-hydroxytryptamine. Like 5-hydroxytryptamine, phenyldiguanide and certain other amidine derivatives caused pain when applied to the base of blisters in human subjects. Unlike 5-hydroxytryptamine, phenyldiguanide did not constrict perfused rat blood vessels or increase the tone of the rat fundal strip preparation of Vane (1957). Phenyldiguanide did not affect the sensitivity of these smooth muscle preparations to 5-hydroxytryptamine, but other amidine derivatives proved to be moderately strong antagonists of the vasoconstrictor actions of 5-hydroxytryptamine and of adrenaline. Unlike 5-hydroxytryptamine, phenyldiguanide did not produce gastric haemorrhage in the mouse. Phenyldiguanide did not prolong chloral hydrate sleeping time in mice by the same mechanism as did 5-hydroxytryptamine. Phenyldiguanide was not highly toxic to mice (LD50 being 240 mg./kg.). It is concluded that phenyldiguanide and certain other amidine derivatives act on sensory receptors which respond to 5-hydroxytryptamine, but that they show little pharmacological resemblance to 5-hydroxytryptamine in other respects.

The chemoreflexes produced by phenyldiguanide (I) and other amidine derivatives were first studied by Dawes and Mott (1950), who concluded that these compounds caused the stimulation of certain sensory receptors in the cardio-pulmonary area. Dawes and Fastier (1950) tested about 100 substances related chemically to phenyldiguanide in the hope that the structural features of compounds found to act like phenyldiguanide might recall those of a substance which could be the natural stimulant of the receptors on which these drugs act. They found that nearly all the more potent compounds were based on the structure consisting of aromatic nucleus–short side chain–unsubstituted amidine group (II).

"Serotonin" had not been fully characterized at the time of this investigation; however, there was evidence that it was a tryptamine derivative. Since it was known that the intravenous injection of serum into cats can cause a reflex fall of blood pressure and heart rate (Brodie, 1900), tryptamine (III) was tested by Dawes and Fastier (1950) and was found to be inactive. However, 5-hydroxytryptamine (IV) produces chemoreflexes which closely resemble those produced by such amidine derivatives as phenyldiguanide (Page, 1952; Comroe, Van Lingen, Stroud and Roncoroni, 1953; Kottegoda and Mott, 1955). It was therefore suggested (Fastier, 1955) that such compounds as phenyldiguanide may resemble 5-hydroxytryptamine sufficiently closely in



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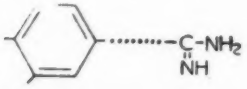

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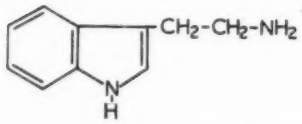

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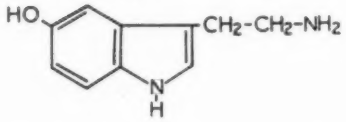

IV

chemical structure for them to be able to stimulate the same chemoreceptors. Evidence bearing on this hypothesis has been obtained in the investigation now to be described.

METHODS

Cats were anaesthetized with chloralose (60 to 70 mg./kg.). Blood pressure was recorded from a carotid artery. The method used for recording respiratory movement was essentially that of Gaddum (1941). Drug solutions were injected into a saphenous vein. Rabbits were anaesthetized with pentobarbitone sodium, guinea-pigs with urethane, and Australian possums (*Trichosurus vulpecula*) with chloralose.

Effects on cutaneous pain receptors in man were studied by the method of Armstrong, Dry, Keele, and Markham (1953). This involves raising a blister on the flexor surface of the forearm, cutting away the separated epidermis to expose the base of the blister, and recording the degree of discomfort estimated by the subject when various solutions are applied to the exposed blister base. A five-point scale was used, no pain being rated 0, slight pain 1, moderate pain 2, fairly severe pain 3, and very severe pain 4. The subject noted the degree of discomfort at 15 sec. intervals. Cantharidin was used as the blistering agent for all the experiments described. However, as one of us developed an extensive papular rash after the third application of cantharidin, we now use a pellet of CO_2 snow in place of cantharidin. Drugs were applied in neutral isotonic solution. The subject was not told what drugs were being tested.

In sleeping time experiments, mice in batches of 10 were given a subcutaneous injection of either an amidine derivative or saline 2 hr. before an intraperitoneal injection of chloral hydrate (250 mg./kg.). Mice were numbered so that individual sleeping times could be accurately assessed. The sleeping mice were kept in boxes at a temperature of 24 to 25°. A cross-over experiment was performed four days later, the mice which previously had been given the amidine derivative being given saline, and vice versa. The sleeping times of individual mice when given chloral hydrate alone were subtracted from their sleeping times when given chloral hydrate together with the amidine derivative. The logarithm of this difference was taken as the variate in statistical tests, since we found that the log. sleeping times of control mice were normally distributed whereas the sleeping times were positively skewed. The rectal temperatures of the mice were measured by inserting a glue-covered constantan-copper thermocouple into the rectum to a fixed distance.

Rat hindquarters were perfused at a constant rate with Ringer-Locke solution as described by Fastier and Smirk (1947). Effects of drugs on the funda portion of the rat's stomach were studied following the technique of Vane (1957).

Compounds other than amidine derivatives were obtained from commercial sources. Most of the amidine derivatives were samples used in previous studies. Specimens of 1-naphthylguanidine, 1-naphthyldiguanide, and 2-naphthyldiguanide were synthesized for us by Mr. L. C. K. Wong. We are indebted to Professor Adrien Albert for the 2-naphthylguanidine.

RESULTS

Effects on the Circulatory System

Reflex Actions

It is now well established that the coronary and allied chemoreflexes are not elicited by phenyldiguanide in vagotomized animals. Even in animals with intact vagi, small intravenous doses of phenyldiguanide do not always elicit sharp falls of blood pressure and of heart rate. This led us to inquire whether those animals which responded atypically to phenyldiguanide would also do so to 5-hydroxytryptamine.

Cats.—Examination of the kymograph records of previous experiments had shown that the responses to phenyldiguanide and 5-hydroxytryptamine are very similar, considering the variation from cat to cat. Typical pairs of responses are illustrated in Fig. 1. As the variation in the course of a single experiment was often considerable, in all the pairs of responses shown the second drug was given within 5 to 8 min. of the first. Predominantly depressor responses were obtained with both phenyldiguanide and 5-hydroxytryptamine in 27 out of 32 cats, almost wholly pressor responses in three others. In two cats the responses to the two drugs differed much more than the paired responses shown in Fig. 1; whereas a small dose of phenyldiguanide produced slight falls of blood pressure, one of 5-hydroxytryptamine produced a slight rise.

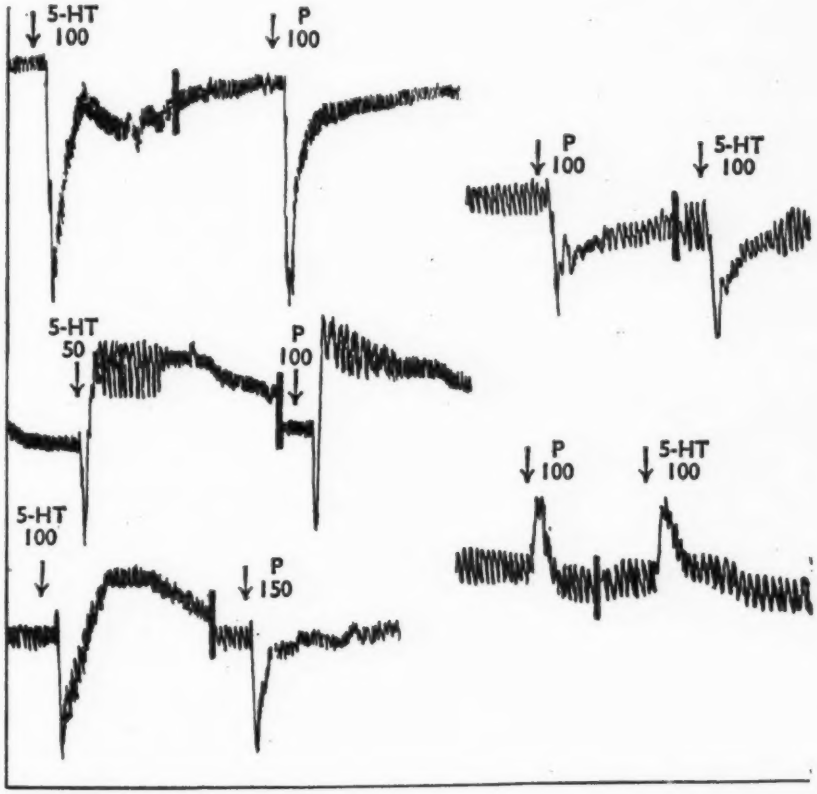

FIG. 1.—Facsimiles of kymograph records showing effects of small doses of 5-hydroxytryptamine (5-HT) and of phenyldiguanide (P) on the arterial blood pressure of anaesthetized cats. Note that the considerable variation from one cat to another in the response to 5-HT is reflected by a similar variation in the response to P. The numerals indicate the dose in μg.

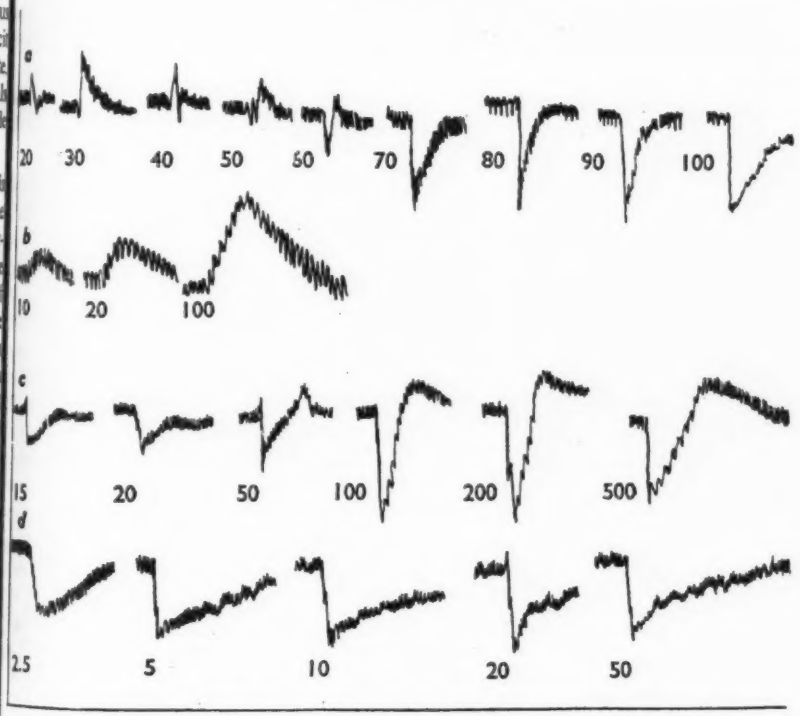

FIG. 2.—Facsimiles of kymograph records showing effects of 5-hydroxytryptamine in graded doses (indicated in μg. by the numerals) on the arterial blood pressure of four anaesthetized cats (*a*, *b*, *c*, and *d*).

In a further series of 18 experiments a wide range of doses was given. In 9 of these experiments doses from 1 to 500 μg. 5-hydroxytryptamine were given. There was a gradual transition from a small pressor to a large depressor response. Fig. 2*a* shows a typical series of responses. Pressor responses only were obtained in 4 out of the other 9 cats (Fig. 2*b*), depressor responses only in the remaining 5 (Figs. 2*c* and *d*).

A pressor element was much less evident in the responses to graded doses of phenyldiguanide. Differences in the cardiovascular effects of the two drugs could therefore be explained by supposing that 5-hydroxytryptamine exerts not only reflex actions practically identical with those of phenyldiguanide, but also an action not shared by phenyldiguanide. While the later phases of the response to 5-hydroxytryptamine differed substantially from those to phenyldiguanide in 12 cats, the initial phases of the responses were usually similar. After vagotomy, pressor responses were generally obtained with 5-hydroxytryptamine, but phenyldiguanide was almost without action on blood pressure.

Other Species. — Reflex depressor responses were readily obtained with both phenyldiguanide and 5-hydroxytryptamine in experiments on rabbits but not on guinea-pigs or Australian possums. The blood pressure of the latter was usually raised by 10 to 100 μg./kg. of 5-hydroxytryptamine, sometimes by more than 50 mm. of mercury, though only for a few minutes. Phenyldiguanide produced small rises of blood pressure in 4 out of 5 possums; the fifth animal responded to both phenyldiguanide and 5-hydroxytryptamine with falls of blood pressure. The pressor responses in other possums were sometimes preceded by slight falls of blood pressure and heart rate. Guinea-pigs resembled possums rather than cats and rabbits in their responses to both drugs.

Interaction with Other Drugs.—In experiments on cats anaesthetized with chloralose, other drugs were given to see if they selectively blocked the reflex depressor actions of 5-hydroxytryptamine and phenyldiguanide.

2-Naphthylguanidine was tested initially because of its chemical resemblance to *S*-2-(2'-naphthyl)ethylisothiourea, the most potent of the amidines examined by Dawes and Fastier (1950). Both 1- and 2-naphthylguanidine, as well as the corresponding diguanides, were found highly active in evoking chemoreflexes. The effects of 2-naphthylguanidine (100 to 200 μg.) were very similar to those produced by somewhat smaller

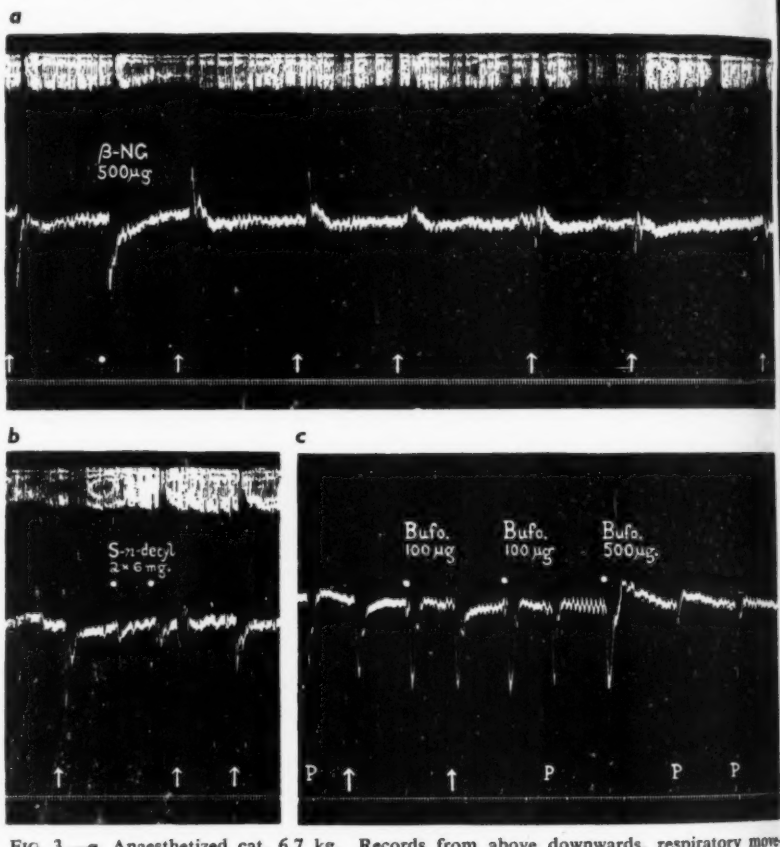

FIG. 3.—**a**, Anaesthetized cat, 6.7 kg. Records from above downwards, respiratory movements, arterial blood pressure (initial level 190 mm. of Hg) and time (10 sec.). Note temporary inhibition of reflex responses to 5-hydroxytryptamine given in 100 μg. doses at each arrow after administration of 500 μg. 2-naphthylguanidine (β-NG). Injections were made at 5 min. intervals. **b**, Continuation of the experiment illustrated in **a**. Note temporary inhibition of reflex effects of 5-hydroxytryptamine given at arrows by *S*-decylisothiourea hydrobromide. (**S-n-decyl**) in two doses of 6 mg. at white dots. **c**, Anaesthetized cat. Note in this experiment that bufotenine (**Bufo.**) in 100 μg. doses produces reflex falls of blood pressure which closely match those produced by 100 μg. doses of phenyldiguanide and 5-hydroxytryptamine (given at P and the arrows respectively), and that a large dose of bufotenine temporarily inhibits the reflex depressor action of phenyldiguanide.

doses of phenyldiguanide in the same cats. After a larger dose of 2-naphthylguanidine (500 μg.) the response of the cat to subsequent doses of phenyldiguanide or 5-hydroxytryptamine was greatly modified for some 10 to 30 min. Fig. 3 shows a typical result: whereas in untreated animals 5-hydroxytryptamine produced typical falls of blood pressure, after 2-naphthylguanidine it caused rises of blood pressure, the response gradually returning to normal as the effect of the guanidine wore off. The response to phenyldiguanide was greatly diminished or even abolished by 2-naphthylguanidine for some 10 to 20 min., but there was no reversal of the action of phenyldiguanide.

Other amidine derivatives, such as 2-naphthyldiguanide and *S*-decylisothiourea, resembled 2-naphthylguanidine in being able to inhibit reversibly the reflex depressor actions of 5-hydroxytryptamine and phenyldiguanide. This is shown for *S*-decylisothiourea in Fig. 3. Blocking of the depressor action of 5-hydroxytryptamine by preceding large doses (up to 1 mg.) of phenyldiguanide could not be clearly demonstrated. When phenyldiguanide and 5-hydroxytryptamine were given at intervals of 5 min. or more, there was no sign of interaction.

Bufotenine was tested like 2-naphthylguanidine for its ability to evoke chemoreflexes. Depressor effects obtained with bufotenine (50 or 100 μg.) in cats closely resembled those with 5-hydroxytryptamine in the same doses. These amounts of bufotenine did not alter the response to 5-hydroxytryptamine or phenyldiguanide given 5 min. later, but 500 μg. of bufotenine substantially diminished the falls of blood pressure obtained with 100 μg. of 5-hydroxytryptamine or phenyldiguanide, though only for 5 to 10 min. This is also shown in Fig. 3.

Procaine had little or no blocking action in doses of 1 to 3 mg./kg.; 10 to 20 mg./kg. abolished the fall of blood pressure obtained with 100 μg. of either 5-hydroxytryptamine or phenyldiguanide.

Adrenaline or *noradrenaline* was infused for 15 to 30 min. at intervals through a jugular vein while test doses of phenyldiguanide and 5-hydroxytryptamine were injected into the saphenous vein. The catechol amines usually raised arterial blood pressure by 50 mm. or more of mercury when given in saline at a rate of 8 to 20 μg./ml./min. In three cats, the depressor effects of phenyldiguanide and 5-hydroxytryptamine were enhanced during the first infusion, and in the remaining four cats they were not much affected. Different results were obtained when adrenaline or noradrenaline was infused for a second or third time, the depressor actions of phenyldiguanide and 5-hydroxytryptamine being either markedly reduced (3 cats) or entirely abolished (4 cats).

Iproniazid or *reserpine* was given to some cats before experiments, as it seemed possible that the response to injected 5-hydroxytryptamine might depend to a large extent upon the amount of endogenous 5-hydroxytryptamine present. Iproniazid is believed to delay the destruction of 5-hydroxytryptamine *in vivo* by inhibiting amine oxidase, and reserpine to deplete the body of 5-hydroxytryptamine and certain other amines. In 5 of 6 cats given two doses of iproniazid (50 mg./kg., the first dose 18 hr. and the second 1 hr. before the main experiment), small intravenous doses of 5-hydroxytryptamine and phenyldiguanide produced falls of blood pressure which did not differ from those obtained in untreated cats. One of the cats pre-treated with iproniazid responded with a rise of blood pressure to small doses of 5-hydroxytryptamine and phenyldiguanide. Pre-treatment with reserpine (2 mg./kg. intraperitoneally 18 hr. before the experiment) likewise failed to produce in anaesthetized cats reflex responses different to those seen in untreated animals.

Other drugs, such as methylamphetamine or pecazine (Pacatal, 10 mg.), temporarily inhibited the reflex depressor actions of 5-hydroxytryptamine and phenyldiguanide.

Lysergic acid diethylamide did not block the reflex depressor action of 5-hydroxytryptamine when given either shortly before it or at the same time in doses of up to 200 μg.

2-(Pentachlorophenoxy)ethylamine was tested because Woolley (1957) has shown that compounds of this type resemble 5-hydroxytryptamine in certain respects. Larger doses (5 or 7 mg.) produced sharp falls of blood pressure and temporary arrest of breathing. The amine did not antagonize the depressor action of 5-hydroxytryptamine to any noteworthy extent, and this action was only partly abolished by vagotomy. This action therefore appears to be due only in part to chemoreflexes.

Actions on Perfused Blood Vessels

In experiments on perfused hind-quarters of the rat, 5-hydroxytryptamine had about 1/10 of the vasoconstrictor activity of adrenaline. Whereas 0.2 μg. of adrenaline was generally sufficient to increase perfusion pressure temporarily by some 60 to 100 mm. Hg, 3 to 5 μg. of 5-hydroxytryptamine was needed to produce a comparable

effect. Phenyldiguanide had no effect on perfusion pressure when tested in doses of from 10 to 100 μg., and it did not modify the response of the perfused vessels to either 5-hydroxytryptamine or adrenaline. Like phenyldiguanide, many of the other amidine derivatives highly effective in evoking chemoreflexes had no effect on vessel tone in doses of up to 100 μg.; however, some of them differed from phenyldiguanide in that they strongly antagonized the vasoconstrictor action of 5-hydroxytryptamine. The active amidines included 2-naphthylguanidine, *S*-(3-phenylpropyl)-isothiourea, and the *o*- and *p*-chloro derivatives of *S*-benzylisothiourea. All these compounds antagonized the action of 5-hydroxytryptamine appreciably when given in a dose not more than 20 times that of 5-hydroxytryptamine, namely 50 μg. of amidine as against 5 μg. 5-hydroxytryptamine. However, the most potent of these compounds had still less than 1/100 of the activity of lysergic acid diethylamide as an antagonist of 5-hydroxytryptamine under the same conditions. Most of the amidines antagonized the vasoconstrictor action of adrenaline about as readily as that of 5-hydroxytryptamine. There was no correlation between potency in reflexly lowering blood pressure in cats and in antagonizing the vasoconstrictor action of 5-hydroxytryptamine.

Effects on the Respiratory System

Previous work, reviewed by Dawes and Comroe (1954), indicates that the effects of phenyldiguanide and 5-hydroxytryptamine on respiratory movement are chiefly due to chemoreflexes evoked by the stimulation of sensory endings in the cardio-pulmonary area. In the cat moderate doses (20 to 50 μg./kg.) usually cause transient apnoea followed by rapid shallow breathing. In other species both agents may produce somewhat different effects.

Their most usual effect in our experiments on guinea-pigs and possums was to inhibit breathing for a few seconds and then stimulate it markedly for several minutes. With these animals, as with cats, a fairly close similarity in the respiratory effects of the two drugs was noted. In support of the idea that they act on the same sensory endings when causing temporary apnoea in the cat is the observation that this effect is inhibited reversibly by 2-naphthylguanidine (see Fig. 3) and by *S*-decylisothiourea.

Effects on Cutaneous Pain Receptors

Armstrong *et al.* (1953) have shown that 5-hydroxytryptamine excites cutaneous pain receptors in man when applied in dilute solution to the exposed blister base. We have used their technique to see if phenyldiguanide and certain other amidine derivatives resemble 5-hydroxytryptamine in this respect.

Experiments were performed on four subjects. They reacted to most drugs in a similar fashion, but several differences were noticed. Thus one subject experienced no pain with acetylcholine even when the concentration was increased to 10^{-3} g./ml. By giving various drug solutions repeatedly, it was possible to gauge the ability of the subject to discriminate between different solutions. Saline or a very dilute solution of a potent drug never produced more than a threshold

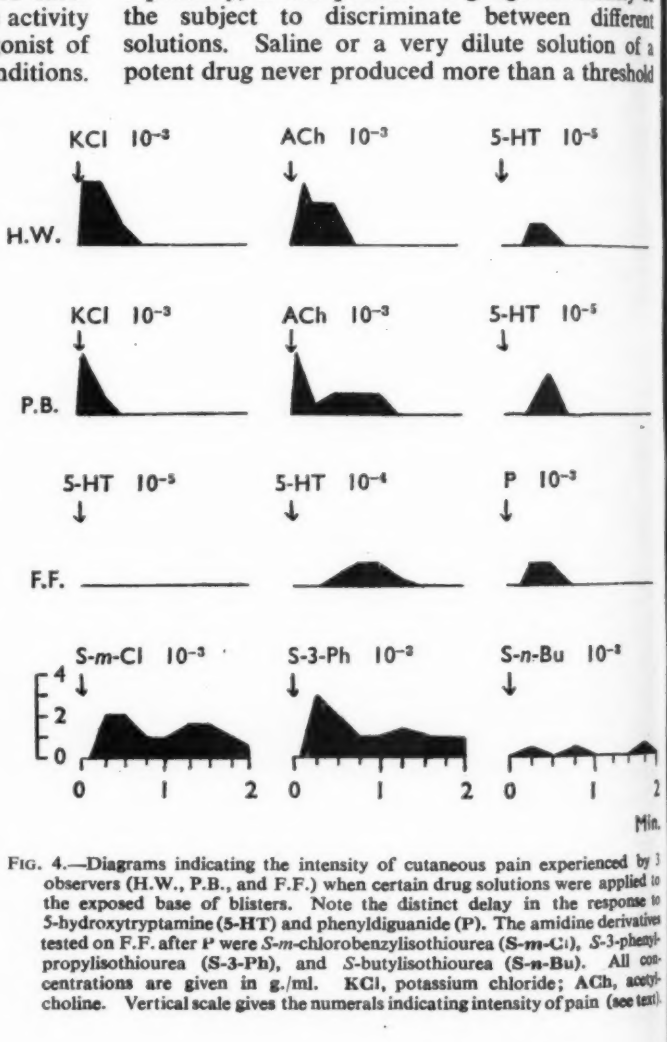

FIG. 4.—Diagrams indicating the intensity of cutaneous pain experienced by 3 observers (H.W., P.B., and F.F.) when certain drug solutions were applied to the exposed base of blisters. Note the distinct delay in the response to 5-hydroxytryptamine (5-HT) and phenyldiguanide (P). The amidine derivatives tested on F.F. after P were *S*-*m*-chlorobenzylisothiourea (S-*m*-Cl), *S*-3-phenylpropylisothiourea (S-3-Ph), and *S*-butylisothiourea (S-*n*-Bu). All concentrations are given in g./ml. KCl, potassium chloride; ACh, acetylcholine. Vertical scale gives the numerals indicating intensity of pain (see text).

effect. Furthermore, the time course of the effect was generally that expected from the previous reaction of the subject, namely an almost immediate response to acetylcholine, histamine, or potassium chloride, but a delayed response to 5-hydroxytryptamine or phenyldiguanide (Fig. 4). However, when some of the drugs were given repeatedly at short intervals, sensitivity to them rapidly diminished. For example, when F. N. F. was given five successive doses of 5-hydroxytryptamine, he experienced considerable pain with the first dose, though the concentration of 5-hydroxytryptamine was only 10^{-8} g./ml., some pain with the second dose, which was 100 times stronger, and subsequently no pain at all with concentrations of 10^{-5}, 10^{-4}, and 10^{-7} g./ml. Yet he remained sensitive to histamine and potassium chloride, though not to tryptamine. Similar results were obtained with the other subjects. Treatment with phenyldiguanide resulted in desensitization to the pain-producing action of 5-hydroxytryptamine. Phenyldiguanide and other amidine derivatives (*S*-benzylisothiourea, *S*-*m*-chlorobenzylisothiourea, *S*-(3-phenylpropyl)isothiourea) resembled 5-hydroxytryptamine in causing delayed pain. A more rapid pain was caused by still other amidine derivatives (guanidine, *asym.*-dimethylguanidine), which do not evoke circulatory and respiratory reflexes in small doses.

Other Effects

Toxicity

Mice that had received 10 mg./kg. of phenyldiguanide intraperitoneally were sedated and showed some respiratory irregularity; breathing was alternately fast and slow. A dose of 20 mg./kg. produced similar effects with hunching of the back. With 50 mg./kg., there was almost immediate respiratory irregularity but less sedation than with the smaller doses. 100 mg./kg. produced similar effects at first; prolonged tachypnoea developed 5 to 10 min. later. When injected intraperitoneally in doses of 230, 250, 280, and 300 mg./kg., phenyldiguanide killed 3, 7, 9, and 10 mice respectively out of batches of 10. Death was usually delayed for an hour or more. It was preceded by utter prostration, laboured breathing, and anoxic convulsions.

Lowering of Rectal Temperature

An intraperitoneal dose of phenyldiguanide (100 mg./kg.) did not lower the rectal temperature of mice to any greater extent than did a control injection of saline. In contrast, 5-hydroxytryptamine lowered rectal temperature considerably even in doses of 10 or 20 mg./kg. When 5-hydroxytryptamine was given by the intracerebral route, a dose of 1 μg. sufficed to lower appreciably the rectal temperature of treated mice, much smaller falls in rectal temperature being obtained with mice that had been given control injections of saline.

Prolongation of Sleeping Time

It has been shown (Fastier, Speden, and Waal, 1957) that 5-hydroxytryptamine prolongs the average sleeping time of mice that have been given a standard dose of chloral hydrate or a barbiturate, probably by lowering body temperature. The results of three experiments with phenyldiguanide are given in Table I. It can be seen that 10 and 50 mg./kg. of phenyldiguanide did not affect sleeping time after chloral hydrate, but 100 mg./kg. produced a highly significant increase.

TABLE I

EFFECT OF PHENYLDIGUANIDE ON CHLORAL HYDRATE SLEEPING TIME

To indicate the broad trend of the results, the geometrical mean for the sleeping times of batches of mice are given in the second column. However, "t" was calculated from log. sleeping times, as described under "Methods."

Dose	Mean Sleeping Time	"t"
10 mg./kg.	21·8 min.	
saline	20·3 ,,	0·40
50 mg./kg.	18·8 ,,	
saline	17·5 ,,	0·28
100 mg./kg.	35·8 ,,	
saline	17·3 ,,	7·2

Actions on Gut

Gastric haemorrhage occurs in mice after large doses of 5-hydroxytryptamine (Blackman, Campion, and Fastier, 1959). Phenyldiguanide or *S*-(3-phenylpropyl)isothiourea (100 mg./kg.) given subcutaneously 6 hr. before the mice were killed and their stomachs examined did not produce such haemorrhages.

Judging by the number and consistency of the faecal pellets after treatment, phenyldiguanide was much less active than 5-hydroxytryptamine in stimulating peristalsis in mice. On the other hand, inspection of the abdominal viscera showed that in rabbits phenyldiguanide, like 5-hydroxytryptamine, caused an increase in peristaltic activity when injected intravenously in small doses (50 to 100 μg./kg.). This effect was less evident in experiments on cats.

The rat fundal strip preparation of Vane (1957), which was contracted by 5-hydroxytryptamine in

doses of as little as 10^{-9} g./ml., was not contracted by phenyldiguanide in concentrations up to 10^{-5} g./ml., nor was the response to 5-hydroxytryptamine affected by the administration of phenyldiguanide.

Action on Striped Muscle

Phenyldiguanide in concentrations of up to 50 μg./ml. did not affect the maximal contractions of the rat diaphragm preparation of Bülbring (1946), stimulated directly or through the phrenic nerve.

Discussion

There is a remarkably close similarity between some of the actions of phenyldiguanide and corresponding actions of 5-hydroxytryptamine. And yet there is no general similarity between the properties of phenyldiguanide and those of 5-hydroxytryptamine.

Points of Difference

Some of the characteristic properties of 5-hydroxytryptamine are largely or entirely lacking in phenyldiguanide. Whereas in our experiments on perfused rat blood vessels 5-hydroxytryptamine constricted blood vessels almost as powerfully as adrenaline, phenyldiguanide did not affect vessel tone. Phenyldiguanide did not raise the tone of rat fundal strips, though these were highly sensitive to 5-hydroxytryptamine. Phenyldiguanide did not produce gastric haemorrhage in the mouse. Moderate doses of phenyldiguanide did not prolong chloral hydrate sleeping time; after a large dose of phenyldiguanide a significant prolongation was seen, but this was not accompanied by a fall of body temperature, such as has been observed in experiments with 5-hydroxytryptamine and which may explain the prolongation by 5-hydroxytryptamine of sleeping time after chloral hydrate (Fastier, Speden, and Waal, 1957). As noted previously (Fastier and Waal, 1957), phenyldiguanide lacks the strong antidiuretic action of 5-hydroxytryptamine.

Points of Similarity

Several types of sensory nerve endings which are stimulated (not necessarily directly) by 5-hydroxytryptamine respond similarly to phenyldiguanide. A resemblance between the two drugs has been noticed in their effects on sensory receptors situated in the heart and lungs (Mott and Paintal, 1953; Dawes and Comroe, 1954; Kottegoda and Mott, 1955; Paintal, 1957), on different types of gastro-intestinal receptor (Paintal, 1954), and on the peristaltic reflex (Bülbring and Lin, 1958). Our experiments in which drugs were applied to the exposed blister base provide evidence for yet another type of sensory receptor which is acted upon by both agents.

The mere fact that amidine derivatives such as phenyldiguanide resemble 5-hydroxytryptamine in producing hypotension, bradycardia and apnoea by reflexes with vagal pathways does not provide sufficient evidence that these compounds are acting at the same sites, for the same triad of effects may be produced by the excitation of various sensory receptors, as emphasized by Dawes and Comroe (1954). Thus, the composite "von Bezold reflex" obtained with typical veratrum alkaloids differs in several important respects from the coronary and allied chemoreflexes elicited by phenyldiguanide. Unlike veratrum alkaloids, phenyldiguanide fails to excite the pulmonary depressor reflex in the dog, though it does so readily in the cat. Again, when transmission through vagal fibres is selectively inhibited by gradual cooling of the vagal trunk, the reflex actions of veratrum alkaloids are blocked long before those of phenyldiguanide (Dawes, Mott and Widdicombe, 1951).

The reflex actions of phenyldiguanide and 5-hydroxytryptamine cannot be differentiated by such means, however. Investigators who have studied both compounds for their reflex actions have been impressed by points of similarity rather than by points of difference. The results of the experiments in which we have compared the reflex depressor effects of 5-hydroxytryptamine and phenyldiguanide under a variety of conditions reinforce the impression that these two drugs are acting at the same sites.

Some Possible Explanations

The very limited pharmacological resemblance of phenyldiguanide to 5-hydroxytryptamine is a strong argument against the possibility that the pharmacological action of phenyldiguanide might be mediated in some way by 5-hydroxytryptamine. Were the effects of phenyldiguanide due to an ability to liberate 5-hydroxytryptamine *in vivo*, then one would expect phenyldiguanide to have the direct as well as the reflex actions of 5-hydroxytryptamine. For the same reason it seems unlikely that phenyldiguanide could act indirectly by potentiating the effects of endogenous 5-hydroxytryptamine. Some of the amidine derivatives which have powerful reflex actions do happen to be powerful inhibitors of amine oxidase (Fastier and Hawkins, 1951). However, the two forms of activity do not run parallel. Further evidence against this idea is

provided by the observation that the reflex effects of phenyldiguanide come on as rapidly as those of 5-hydroxytryptamine and are as evanescent.

Both drugs might act on the same cells but in fundamentally different ways. The gross reactions of a nerve cell are probably few. It is not unlikely that the same end-effects could be produced by drugs which acted on different parts of the same cells or which affected different chemical reactions going on there.

The alternative hypothesis, that phenyldiguanide acts in essentially the same way as 5-hydroxytryptamine at certain sensory nerve endings, implies that the different types of cellular " receptor " for 5-hydroxytryptamine differ so markedly from one another that phenyldiguanide is able to affect only certain of them.

The receptors on which 5-hydroxytryptamine can act may well differ somewhat from one to another as regards accessibility or configuration. Different types of receptor have already been postulated for 5-hydroxytryptamine (Gaddum and Hameed, 1954), as they have been also for adrenaline (Ahlquist, 1948) and yet other agents.

Blocking Agents.—Perhaps the most convincing evidence that phenyldiguanide and 5-hydroxytryptamine act on the same receptors is provided by the experiments with 2-naphthylguanidine and with bufotenine. It did not prove possible to antagonize the reflex depressor action of 5-hydroxytryptamine by giving one of these blocking agents without producing a similar modification of the response to phenyldiguanide. The blocking action of 2-naphthylguanidine is probably exerted on those sensory receptors which are stimulated by phenyldiguanide, since there is chemical similarity between the compounds and since 2-naphthylguanidine acts very much like phenyldiguanide when it is given in smaller doses than those having a blocking action. Similarly, the blocking action of bufotenine is probably exerted on those sensory receptors which are stimulated by 5-hydroxytryptamine. We therefore think it significant that the reflex depressor actions of both phenyldiguanide and 5-hydroxytryptamine can be blocked by giving *either* 2-naphthylguanidine or bufotenine. Presumably, all four compounds have a common site of action, but only 2-naphthylguanidine and bufotenine form sufficiently stable combinations with the receptors to prevent further excitation for a considerable period. Blocking of reflex actions has been observed previously with *S*-nonylisothiourea and with 5-amino-3-ethyl-2-methylindole (Dawes and Comroe, 1954). It seems likely that these effects are closely similar to those described in the present work.

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THE ACTIONS OF BRETYLIUM: ADRENERGIC NEURONE BLOCKING AND OTHER EFFECTS

BY

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Bretylium caused a specific and lasting depression of many excitatory and inhibitory responses evoked by electrical stimulation of the peripheral sympathetic nervous system, probably by impairing conduction of impulses in adrenergic neurones with consequent failure of noradrenaline and adrenaline release. This effect, which will be referred to as the adrenergic neurone blocking action, was preceded by weak sympathomimetic effects. In the presence of bretylium the effects of adrenaline and noradrenaline were increased, as after sympathectomy. Concentrations producing blocking of adrenergic neurones did not prevent the release of adrenaline and noradrenaline from the adrenal medulla by splanchnic nerve stimulation or by the injection of dimethylphenylpiperazinium iodide, nor did they cause antiparasympathetic or parasympathomimetic effects. No action on the central nervous system has been detected. Curare-like neuromuscular block occurred with 10 to 30 times the amount required to block the response to adrenergic nerve stimulation alone and was accompanied by signs of temporary synaptic block in autonomic ganglia. Adrenergic nerve trunks and sensory nerves in the skin were readily blocked for long periods by topical application of bretylium, whereas the phrenic nerve of the rat was not. Bretylium had little effect on gastrointestinal propulsion or on the sensitivity of smooth muscle to acetylcholine, 5-hydroxytryptamine, adrenaline, or noradrenaline, but moderate amounts depressed the peristaltic reflex and the sensitivity of the guinea-pig ileum to histamine. Bretylium caused postural hypotension in the cat in doses which had little effect on the supine blood pressure. Experiments on the nictitating membrane indicated that compensation for the effects of bretylium on low rates of stimulation of postganglionic sympathetic nerves could be attained by a small increase in the rate of stimulation, whereas compensation for its effects on high rates required an increase in the rate of stimulation beyond physiological limits.

The properties of choline 2,6-xylyl ether bromide, TM 10 (Hey and Willey, 1954; Exley, 1957; Bain and Fielden, 1957), suggested that compounds might be found which would selectively impair the function of adrenergic nerves without antagonizing noradrenaline and adrenaline. A search for such compounds revealed a number of quaternary benzylammonium salts that specifically blocked the effects of stimulating adrenergic nerves, but lacked the parasympathomimetic actions associated with choline 2,6-xylyl ether bromide (Boura, Copp, and Green, 1959). One of the most active and selective of these compounds was the *N*-*o*-bromobenzyl-*N*-ethyl-*NN*-dimethylammonium ion known as bretylium. Trials have shown that bretylium lowers the blood pressure in normotensive and hypertensive men, and that its effects are confined to the peripheral sympathetic nervous system (Boura, Green, McCoubrey, Laurence, Moulton, and Rosenheim, 1959).

This paper contains a description of the acute effects of bretylium, because only brief reports of the pharmacological properties of the drug have been published.

METHODS

Experiments in Vitro.—The preparation described by Finkleman (1930) was used to study the effects of the drug on the depressant action of postganglionic sympathetic nerve stimulation on the rabbit intestine. The nerve was stimulated through platinum electrodes, with supramaximal shocks at 50/sec. A similar procedure was used to examine the action of the drug on contractions of isolated rabbit uterus caused by stimulation of the hypogastric nerve. Usually drugs were added to the bath fluid, but in some experiments they were applied topically to the nerve by introducing the drug dissolved in Tyrode solution into a small

chamber, sealed with rubber dams at each end, through which the nerve had been drawn. Isolated preparations were also used to examine the effect of the drug on the actions of various local hormones. Tyrode solution was used for guinea-pig and rabbit intestine, Locke solution for guinea-pig uterus, a modified Tyrode solution containing 0.02% $MgCl_2$ for rabbit uterus, and the solution described by Gaddum, Peart, and Vogt (1949) for rat uterus. These solutions were oxygenated and maintained at 37°.

Rabbit ears were perfused with Tyrode solution at 37°, and a Thorp impulse counter measured venous outflow. Vasoconstriction was produced by adding drugs to the perfusion fluid or by stimulating the greater auricular nerve adjacent to the medial artery.

Actions at the neuromuscular junction were tested on the rat diaphragm-phrenic nerve preparation (Bülbring, 1946). Single rectangular pulses of 0.7 msec. duration were applied to the nerve through fluid electrodes at the rate of 5/min.

Experiments on Anaesthetized Cats.—Anaesthesia was induced with ether and maintained with chloralose (about 60 mg./kg., intravenously). In dogs, pentobarbitone sodium (30 to 40 mg./kg.) was injected intravenously. Blood pressure was recorded from a carotid artery and injections were usually made into a femoral vein. Contractions of the nictitating membrane were recorded using an isotonic frontal-writing lever. Contractions of the heart were recorded with a Cushny myocardiograph attached to an isometric writing lever which, by closing a mercury switch, operated a Thorp impulse counter, so recording the heart rate.

Various nerves were cut and drawn into fluid electrodes of the type described by Porter and Allamon (1936). They were usually stimulated at 10 to 20 pulses/sec. However in experiments on the nictitating membrane (Fig. 5) various other frequencies were applied to the postganglionic nerve; each of five stimulation rates was applied for 2 min. to the nerves supplying each of the two membranes, five times in random order, and the mean height of contraction determined for each frequency. This was done at the beginning of the experiment, again 10 min. after each of a series of progressively increasing doses of bretylium and finally after the injection of cocaine.

The superior cervical ganglion of the cat was perfused in the manner employed by Perry (1953).

The effect of bretylium on the noradrenaline content of the venous blood from the spleen during stimulation of the splenic nerve was studied by the method of Brown and Gillespie (1957). The nerve was stimulated with 25 pulses/sec. The pressor activity of the plasma was assayed in terms of noradrenaline in the pithed rat.

The position of the cats was supine, except in postural hypotension experiments in which the animals were tilted through approximately 75° until the blood pressure fall was maximal.

Experiments in Unanaesthetized Animals.—Changes in sympathetic tone in the cat were observed by measuring the portion of the nictitating membrane exposed at the lower lid margin. Pupil diameters in mice were measured using a ×10 magnifying lens fitted with a scale. Effects on gastrointestinal propulsion in the rat were examined by the charcoal meal test (Green, 1959).

Local anaesthesia was tested by pricking the skin with a fine needle after intradermal injection in man and guinea-pig. In the latter species, the method of Bülbring and Wajda (1945) was used.

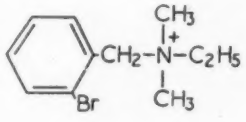
Activity of mice was recorded in individual jiggle cages which were sensitive to limb movements. The mice were placed in the jiggle cage for counting periods of 10 min., starting 15 min. after 50 mg./kg. of bretylium in the first test, and starting 2, 4, and 6 hr. after 2.5, 5, 10, 20, and 40 mg./kg. intraperitoneally in a second test. In each experiment six cages were used in each of six runs, and treatments were randomized between them. In the second test, five groups of six mice received the various doses of bretylium and a control group saline. The mean control counts were 148, 112, and 110/min. at 2, 4, and 6 hr. respectively.

An analgesic action was sought by comparing the pain thresholds of a group of ten rats given 50 mg./kg. subcutaneously with those of a control group injected with saline. The thresholds were measured after ½, 1, 2, 4, and 6 hr. by the pressure and heat methods described by Green and Young (1951).

The intravenous, subcutaneous and oral LD50 values in mice were approximately 81 to 125, 81 to 123, and 53 to 190% of the means respectively ($P=0.05$).

Before the administration of drugs by stomach tube, mice and rats were allowed water *ad libitum* but had received no solid food for 12 to 18 hr. Cats were restricted to milk and water for 24 hr. and were lightly anaesthetized with ether for the drug administration.

Atropine was given as the sulphate, *N'*,*N'*-dimethyl-N²-phenylpiperazinium as the iodide, and cocaine as the hydrochloride. The amounts of bretylium refer to the bromide unless the tosylate (Darenthin) is specifically mentioned. The molecular weights of bretylium bromide and bretylium tosylate are 341 and 414 respectively. The structure of bretylium is:



RESULTS

Effects on the Peripheral Sympathetic Nervous System

Rabbit Ileum. — The inhibitory effect of stimulating the visceral nerve on the pendular movements of isolated rabbit ileum was abolished after adding bretylium in concentrations of 1 to

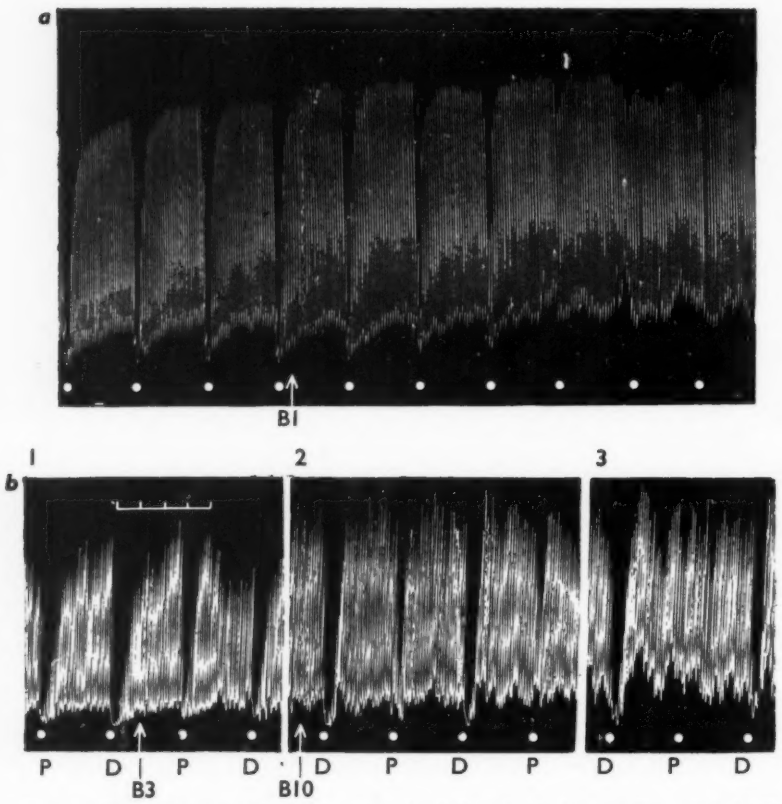


FIG. 1.—The effect of adrenergic nerve stimulation decreasing motility of isolated rabbit ileum. Supramaximal stimulation at white dots (50 pulses/sec. for 20 sec. every 3 min.). *a*, Bretylium was added to the organ bath in a concentration of 1 μg./ml. (at **B1**). *b*, Bretylium was applied to the nerve only in concentrations of 3 and 10 μg./ml. (**B3** and **B10** respectively). P, Stimulation proximal to and D distal to the nerve bath. Between records ***b*1** and ***b*2**, 15 min., and between ***b*2** and ***b*3**, 30 min.

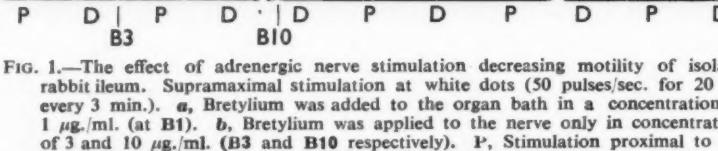


FIG. 2.—Contractions of isolated rabbit uterus caused by stimulation of the hypogastric nerve. The vertical lines indicate supramaximal stimulation (50 pulses/sec. for 1 min.). From **B10**, the nerve bath contained bretylium at 10 μg./ml. and from **B50** it contained 50 μg./ml. Time, 1 min.

3 μg./ml. to the bath fluid (Fig. 1*a*). With these concentrations the effect took about 20 min. to develop fully, but higher concentrations acted more rapidly. The effect persisted after washing the tissue several times. The inhibitory effect of bretylium was reduced by adding cocaine (100 μg./ml.). The response to nerve stimulation could also be abolished by immersing the nerve in bretylium (10 to 100 μg./ml.) between the point of stimulation and the intestine (Fig. 1*b*). It was important to choose a preparation without much fat around the nerve.

The inhibitory effects of adrenaline and noradrenaline (10 to 100 ng./ml.) on the pendular movements of isolated rabbit ileum were materially unaltered by previous treatment with bretylium (30 μg./ml.).

Rabbit Uterus.—Stimulation of the hypogastric nerve supplying an isolated horn of the rabbit uterus caused contractions. This response to nerve stimulation was abolished by concentrations of 3 to 10 μg./ml. of bretylium in the bath fluid or by adding bretylium (10 to 50 μg./ml.) to the Ringer solution in the nerve bath situated between the point of stimulation and the muscle (Fig. 2). The sensitivity of the uterus to 20 ng./ml. of adrenaline or noradrenaline was not reduced unless large concentrations of bretylium (100 μg./ml.) were added to the bath.

Vasodilatation in the Rabbit.—The subcutaneous injection of 10 mg./kg. of bretylium was followed by vasodilatation, which was seen most readily in the ears. The vasoconstriction in perfused rabbit ear, caused by stimulation of the greater auricular nerve, was blocked by the injection of 30 to 100 μg. of bretylium into the perfusion fluid (Fig. 3*a*) or by local application to the nerve of a concentration of 100 μg./ml. between the stimulating electrodes and the ear. In contrast, the vasoconstriction following the injection of 0.1 to 1.0 μg. of noradrenaline into the perfusion fluid

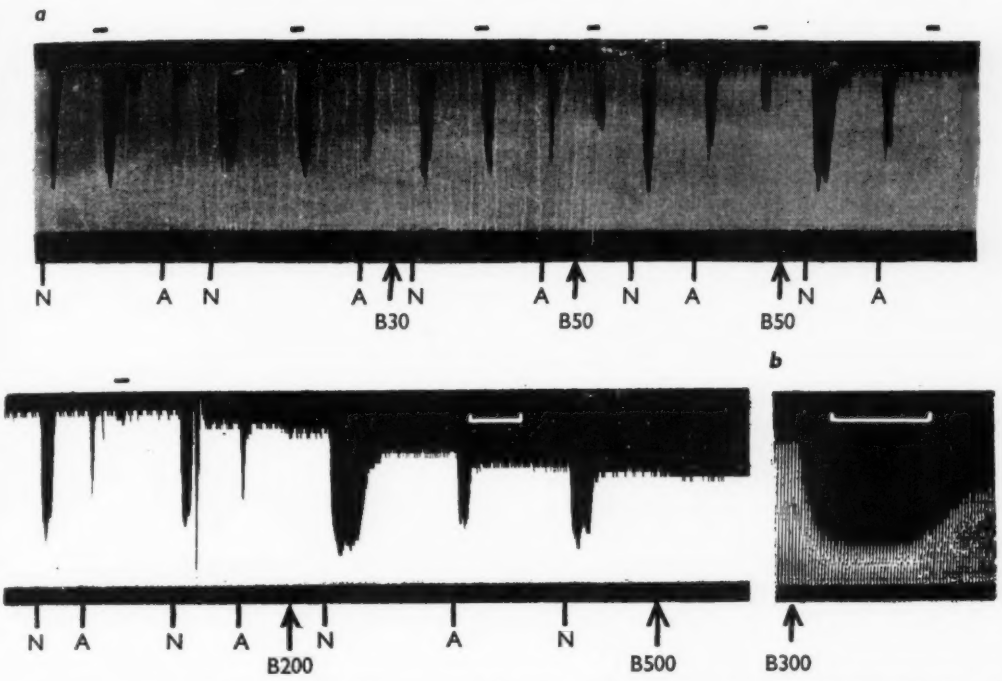


FIG. 3.—Venous outflow from a perfused rabbit ear, recorded with a Thorp impulse counter. The horizontal bars indicate supramaximal stimulation of medial nerve (50 pulses/sec. for 1 min.). At **N**, 0.2 μg. of noradrenaline, and at **A**, 0.1 μg. of adrenaline were injected into the arterial cannula. At **B30**, **B50**, **B200**, **B500**, and **B300**, 30, 50, 200, 500, and 300 μg. of bretylium respectively were injected into the arterial cannula. Records *a* and *b* were made from different ears. Time, 5 min.

was increased after the injection of 50 to 300 μg. of bretylium (Fig. 3*a*). The response to adrenaline (0.1 to 1.0 μg.) was increased to a lesser extent and, in some experiments, was unchanged. Vasoconstriction occurred after injecting large amounts of bretylium (100 to 300 μg.) into the perfusion fluid. In most experiments the response was similar to that in Fig. 3*b*.

Nictitating Membrane. — The subcutaneous injection of 5 to 10 mg./kg. bretylium into cats caused relaxation of the nictitating membrane and narrowing of the palpebral fissure. The pupils became smaller but dilated in dim light. The relaxation of the nictitating membrane was the most characteristic effect. Usually it did not appear until 3 hr. had elapsed and was greatest between 12 and 24 hr. Normal tone of the membrane did not usually return for 36 to 48 hr. When the drug was administered by stomach tube, larger amounts of bretylium were needed to produce these effects. The onset of action tended to be slower and the duration longer with oral than with subcutaneous injections (Fig. 4). In cats in which the membranes had been relaxed by administration of bretylium, the injection of adrenaline, noradrenaline or a further amount of bretylium caused transient contractions of the membranes.

The nictitating membranes of dogs were also relaxed by injection of bretylium, in amounts similar to those which were effective in cats. Again the effect was slow in onset and persisted at least 24 hr.

In cats anaesthetized with chloralose, the nictitating membrane retracted after the intravenous injection of large amounts of bretylium. The effect was small or absent after 5 mg./kg. or less, but 10 to 20 mg./kg. often caused retractions ranging between 20 and 80% of those due to maximal stimulation of the cervical sympathetic nerve. The action persisted after cutting the pre- or post-ganglionic nerves, and removal of the adrenals had little effect. Retraction of the membrane was often maintained for over 1 hr. A second injection of drug given when the membrane had relaxed caused a temporary contraction. These findings accord with those observed in unanaesthetized animals.

After administration of bretylium the nictitating membrane failed to maintain the tonic retraction

in response to electrical stimulation of the cervical sympathetic nerve, irrespective of whether the stimulation was applied proximally or distally to the superior cervical ganglion. High rates of stimulation were affected to a relatively greater extent than were low rates of stimulation, as is seen in Fig. 5. The responses of the membranes on each side were recorded during continuous stimulation of both the postganglionic nerves at each of five frequencies five times in random order. Bretylium (2 to 6 mg./kg.) reduced the regression of response on the log. of the frequency of stimulation and, after a total dose of 10 mg./kg. of bretylium, the sensitivity to all rates of stimulation was practically abolished. Such membrane tone seen in Fig. 5 after 10 mg./kg. was largely accountable to the effect of bretylium itself. After bretylium had depressed the response of the nictitating membrane to nerve stimulation, resting the preparation was followed by a temporary increase of the response. The intravenous injection of adrenaline, noradrenaline or dimethylphenylpiperazinium in the non-adrenalectomized cat continued to cause contractions of the membrane after administration of bretylium in amounts sufficient to abolish its response to nerve stimulation.

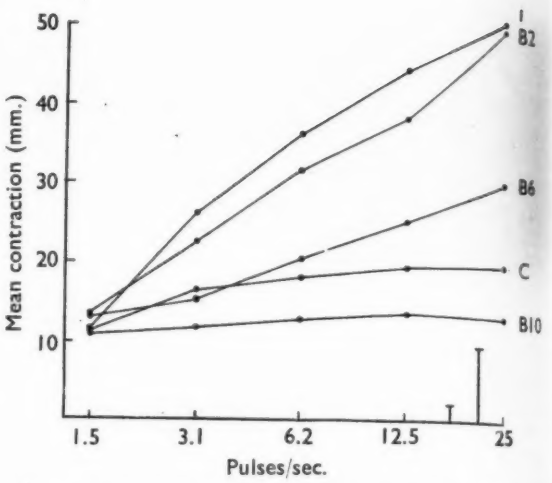


FIG. 5.—Contractions of the nictitating membranes of a cat under chloralose anaesthesia during stimulation of the postganglionic cervical sympathetic nerves at various frequencies by supra-maximal rectangular pulses. Each point represents the mean of five responses on each of the two membranes. **I**, Initial; **B2**, after 2 mg./kg. of bretylium intravenously; **B6**, after a total of 6 mg./kg.; **B10**, after a total of 10 mg./kg.; **C**, after 1 mg./kg. of cocaine in addition. The vertical lines represent the heights of the sustained contractions of the membranes caused by 6 and 10 mg./kg. of bretylium respectively in the absence of nerve stimulation.

The intravenous injection of 1 mg./kg. of cocaine reduced the effect of intravenous bretylium on the membrane response to nerve stimulation (Fig. 5). In other experiments when 1 mg./kg. of cocaine reduced the depressant effect of 5 mg./kg. of bretylium on the response to nerve stimulation, the response to injected adrenaline was increased to a similar extent (Fig. 6). Increasing the amount of cocaine administered caused no further increase in either response whereas, at this stage, a further dose of 5 mg./kg. of bretylium depressed the response to nerve stimulation but not that to adrenaline.

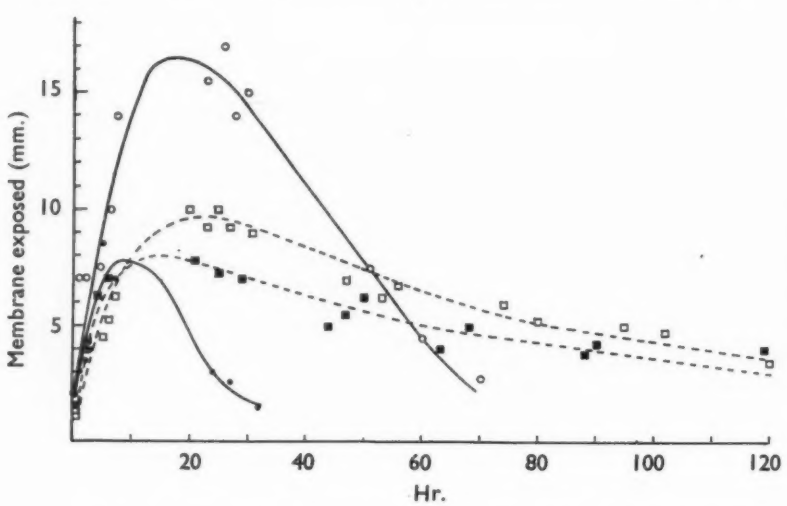


FIG. 4.—Relaxation of the nictitating membrane after bretylium in the unanaesthetized cat. Each point is the mean of observations on four animals. The higher oral and subcutaneous doses were maximal in effect for the method of administration. O——O, 10 mg./kg. subcutaneously. ●——●, 2.5 mg./kg. subcutaneously. □----□, 50.0 mg./kg. orally. ■----■, 12.5 mg./kg. orally.

The injection of 1 mg. of bretylium into the carotid artery of an adrenalectomized cat (3.8 kg. body weight) caused a small retraction of the nictitating membrane (10% of maximal) lasting about 3 min., and 15 min. later it had reduced the response to stimulating the postganglionic nerve by about 50%. When a further 2 mg. was given the membrane retracted to about 20% of maximum and regained its normal tone after about 15 min. This quantity reduced the response to nerve stimulation by 95%. A further 3 mg. produced a similar degree of membrane

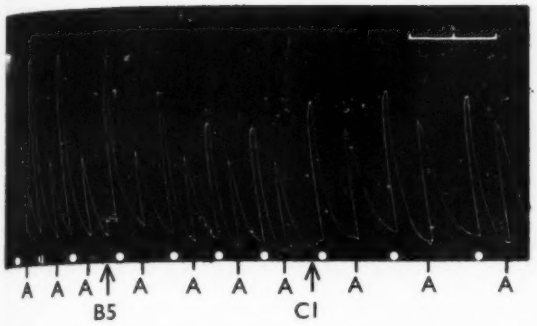


FIG. 6.—Contractions of the nictitating membrane of a cat anaesthetized with chloralose. Supramaximal rectangular pulse stimuli 25/sec. were applied to the postganglionic nerve for 30 sec. at the white dots. **A**, 50 μg. of adrenaline intravenously; **B5**, 5 mg./kg. of bretylium intravenously; **C1**, 1 mg./kg. cocaine intravenously. Time, 10 min.

retraction lasting 25 min. and eliminated the response to nerve stimulation.

In the perfused superior cervical ganglion preparation in the cat, injection of 100 μg. of bretylium caused a small temporary retraction of the nictitating membrane followed by a temporary impairment of the effects of preganglionic stimulation. The addition of bretylium to the perfusion fluid depressed the response to preganglionic nerve stimulation in the manner characteristic of ganglionic blocking agents (Fig. 7). Return to a perfusion fluid containing no bretylium led to a full restoration of the membrane retractions.

In an adrenalectomized cat which had received atropine (2 mg./kg.), intravenous injection of 5 or 10 mg. of acetylcholine caused retractions of the acutely denervated nictitating membranes which, though diminished, continued after 9 mg./kg. of bretylium but ceased after a further 3 mg./kg. of atropine.

Cardiovascular Effects.—The blood pressure of supine cats under chloralose anaesthesia usually increased temporarily after the intravenous injection of 3 to 10 mg./kg. of bretylium and then, after a period of 2 to 10 min., gradually fell. The rise in pressure varied considerably in different cats, but seldom exceeded 30 mm. Hg after 3 mg./kg. of bretylium or 60 mm. Hg after 10 mg./kg. It was accompanied by tachycardia. The delayed lowering of blood pressure was so gradual that in experiments in the anaesthetized animal we could not always be sure that the effect was distinct from a possible deterioration of the preparation. However, in most experiments the pressure had fallen 30 to 60 mm. Hg below the pre-injection level within 30 to 60 min. after injection of 5 to 10 mg./kg. A return to pre-injection levels was never attained in any experiment, but some recovery was observed within 6 hr. on two occasions. The pressor effect of bretylium was greatest in animals with a low blood pressure, as in the spinal preparation. It also persisted after ganglionic blockade or adrenalectomy.

Postural hypotension was produced by bretylium in doses below those which materially changed the supine pressure. When supine cats were tilted towards the vertical position, the mean carotid blood pressure usually fell about 20 mm. Hg. After 1 to 3 mg./kg. of bretylium, the postural fall was as much as 50 to 100 mm. Hg (Fig. 8). Cardio-acceleration caused by stimulation of the inferior cardiac nerve was

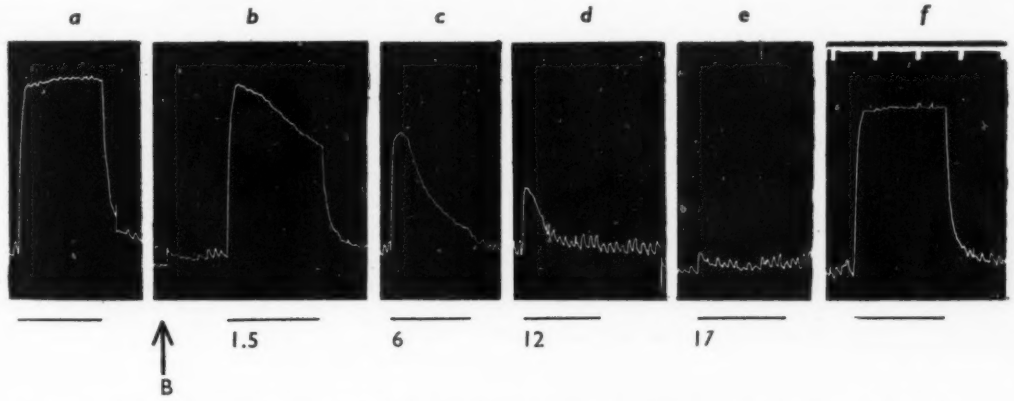


FIG. 7.—The effect of bretylium added to the fluid perfusing the isolated superior cervical ganglion of the cat. Record of the nictitating membrane responses caused by supramaximal stimulation of the preganglionic cervical sympathetic nerve (10 pulses/sec.). The presence of continuous stimulation is indicated by the horizontal lines. ***a***, Contraction before the drug. ***b*** to ***e***, contractions during the perfusion with Tyrode solution containing 100 μg./ml. of bretylium started at **B**; the numerals give the time in minutes from changing to this solution. ***f***, Contraction 8 min. after returning to normal Tyrode solution. Time, 1 min.

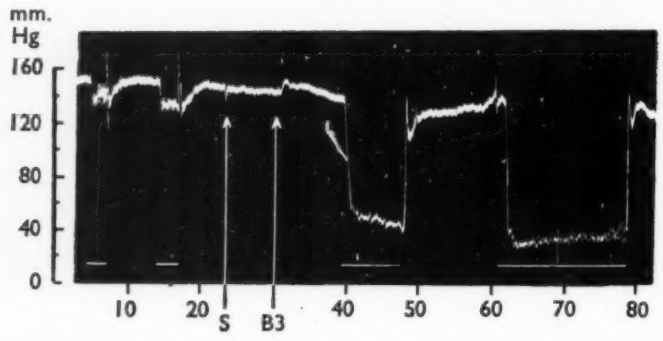


FIG. 8.—Postural hypotension after bretylium in a cat anaesthetized with chloralose. The cat was supine except when tilted through 75°, for the periods indicated by the horizontal white lines. Saline was injected intravenously at **S** and 3 mg./kg. of bretylium at **B3**. The numerals **10** to **80** indicate the time in min.

gradually abolished after the intravenous injection of 1 to 3 mg./kg. of bretylium (Fig. 9). When bretylium had blocked the cardiac acceleration in such experiments, stimulation of the nerve caused some slowing of the heart which continued after increasing the bretylium dose to 10 mg./kg. but was abolished by 1 mg./kg. of atropine.

Bretylium also reduced the reflex hypertension caused by stimulating the central end of the cut vagus nerve and the hypertension caused by carotid occlusion in the cat, the minimal effective intravenous dose being about 3 mg./kg. Further, the pressor effect of intravenous acetylcholine (5 mg.) in an atropinized adrenalectomized cat was abolished by 3 mg./kg. bretylium. On the other hand, the pressor action of intravenous noradrenaline, and to a lesser extent that of adrenaline, increased after giving sufficient bretylium to block adrenergic nerve transmission, and adrenaline continued to cause tachycardia (Fig. 9).

In a dog, 3 mg./kg. of bretylium intravenously caused hypertension lasting about 20 min. The pressor response to carotid occlusion was abolished but that to noradrenaline was increased.

The Spleen and its Noradrenaline Output.—Contraction of the spleen caused by bursts of stimuli at 25/sec. applied to the splenic nerve ceased after injection of 5 to 10 mg./kg. of bretylium. This was accompanied by a reduction in the noradrenaline content in the venous blood from the spleen. Fig. 10 shows the amount of noradrenaline released by each stimulus to the nerve, in each of two experiments.

Sweat Glands.—Bretylium in intravenous doses of up to 10 mg./kg. did not prevent sweating of the paws of anaesthetized cats following stimulation of the peripheral sympathetic chain.

Adrenal Gland.—No change in the functioning of the adrenal medulla was observed after the administration of bretylium. In cats, splanchnic nerve stimulation continued to exert a pressor effect even after 10 mg./kg. of the drug (Fig. 11). In none of three cats was there a rapid component in the hypertensive response attributable with certainty to contraction of the splanchnic vessels, but in the one cat where the record indicated a likelihood of its presence, the rapid component disappeared after giving bretylium.

Intravenous doses of 20 to 50 μg. of dimethylphenylpiperazinium cause a liberation of catechol amines from the adrenal medulla in the cat, and the hypertension so produced was enhanced by bretylium (Fig. 11), like that of injected adrenaline or noradrenaline. After adrenalectomy larger doses of dimethylphenylpiperazinium (100 μg.) are needed to cause

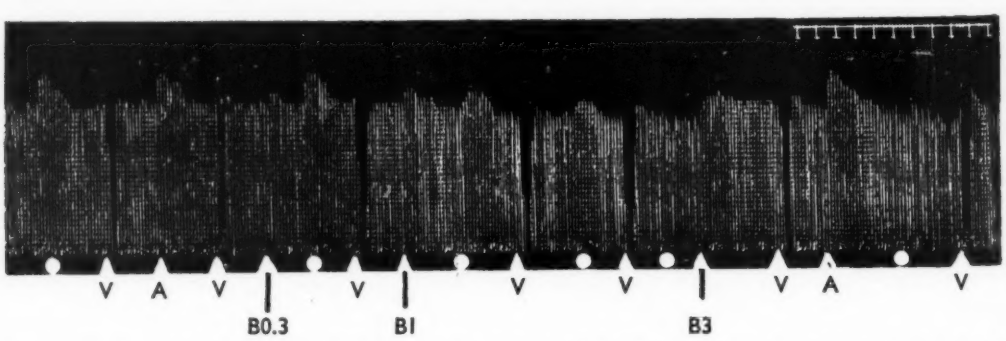


FIG. 9.—The heart rate of a cat anaesthetized with chloralose recorded with a Thorp impulse counter. Bretylium (0.3, 1.0, and 3 mg./kg.) was injected intravenously at **B0.3, B1,** and **B3** respectively. It abolished the cardioacceleration caused by supramaximal stimulation of the inferior cardiac nerve for 30 sec. with 20 pulses/sec. (at white dots), but not the responses to vagal stimulation for 20 sec. at 20/sec. (at **V**). The response to 5 μg. of adrenaline intravenously (at **A**) was unchanged. Time, 1 min.

hypertension, and this effect (presumably due to stimulation of the sympathetic ganglia) was antagonized by 3 mg./kg. of bretylium (Fig. 12), like that of acetylcholine in the atropinized adrenalectomized cat.

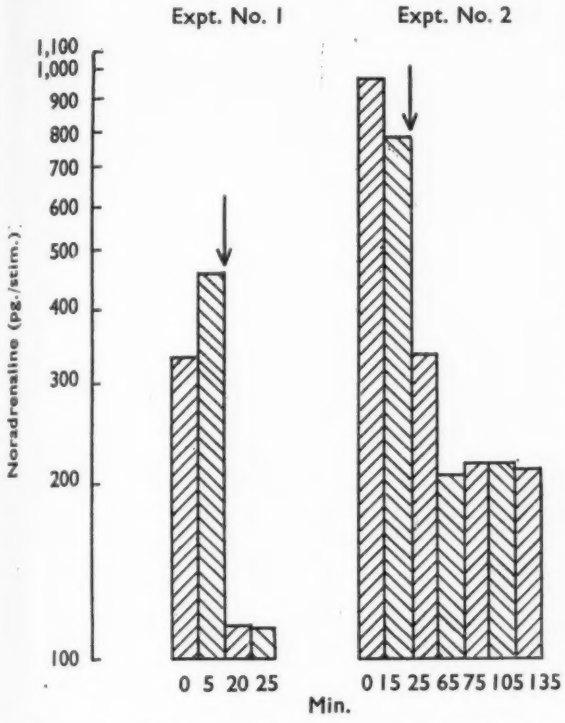


FIG. 10.—The effect of bretylium on the noradrenaline content of the venous blood from the spleen during stimulation of the splenic nerve. Stimuli were applied to the nerve at the rate of 25/sec. for 10 sec. in Expt. No. 1 and for 60 sec. in Expt. No. 2. The venous blood was collected for 30 sec. from the beginning of stimulation in Expt. No. 1, and for 2 min. in Expt. No. 2. The output of noradrenaline, assayed in the pithed rat, is expressed in pg./stimulus. The arrows indicate the intravenous injection of 10 mg./kg. of bretylium in Expt. No. 1 and 5 mg./kg. in Expt. No. 2.

Actions Associated with the Parasympathetic Nervous System

No effects attributable to an action on the peripheral parasympathetic nervous system have been observed, except with high concentrations of bretylium. Large, near toxic, intravenous doses caused a number of transitory effects resembling those produced by ganglionic blocking agents. In cats, 15 mg./kg. intravenously dilated the pupil and abolished its response to light for a few minutes. The nictitating membrane temporarily relaxed, but this was distinct from the later, slowly developing, persistent effect on the membrane, which we associate with a depression of the adrenergic nerve function. No mydriasis was seen after amounts of up to 30 mg./kg. subcutaneously in cats. In mice given 12.5 mg./kg. intravenously, the pupils measured under a powerful light source rapidly dilated to about half the diameters found after fully effective doses of ganglionic blocking agents, but returned to the control size in 5 to 10 min. Mydriasis was not seen after 50 mg./kg. intraperitoneally in mice, nor after 15 mg./kg. intramuscularly in dogs. Similarly, after the intravenous injection of 10 mg./kg. in the cat anaesthetized with chloralose, the bradycardia caused by stimulating the distal end of the cut vagus was reduced, but only for a few minutes. The depressor effect of intravenous acetylcholine was not appreciably changed.

Bretylium had no effect on the tone of the isolated guinea-pig or rabbit ileum except in very high concentrations. In a comparative test, 200 μg./ml. caused a spasm of guinea-pig ileum equivalent to those caused by 40 ng./ml. of

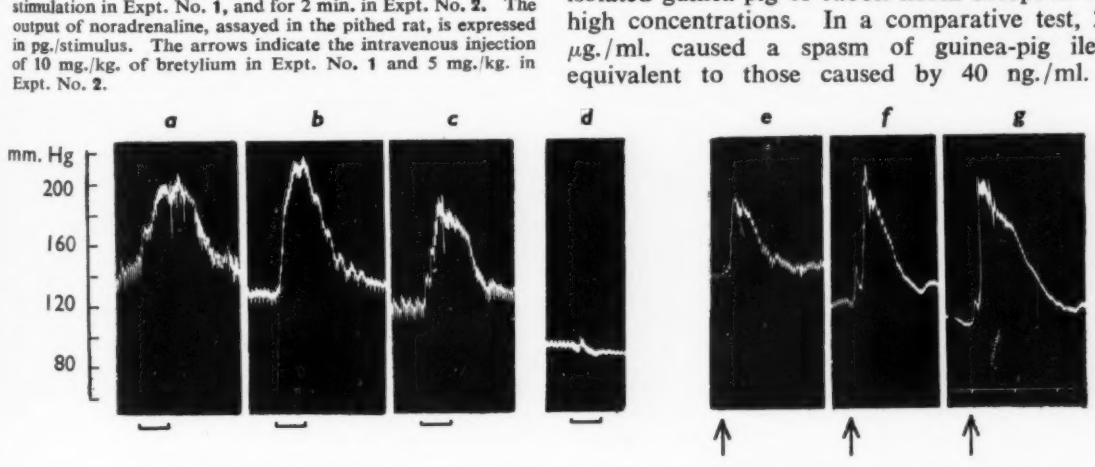


FIG. 11.—Records of the blood pressure of cats anaesthetized with chloralose. The first group shows the pressor responses to supramaximal stimulation of the splanchnic nerve at 15/sec. for 40 sec. (horizontal bars). *a*, Before the drug; *b* and *c*, 50 min. after 3 and 10 mg./kg. of bretylium intravenously respectively; *d*, 8 min. after 1 mg./kg. pentacynium intravenously. The second group shows the pressor responses to 20 μg. dimethylphenylpiperazinium intravenously in another cat (at arrows). *e*, Before the drug; *f*, 35 min. after 3 mg./kg.; and *g*, 45 min. after 10 mg./kg. of bretylium. Time, 1 min.

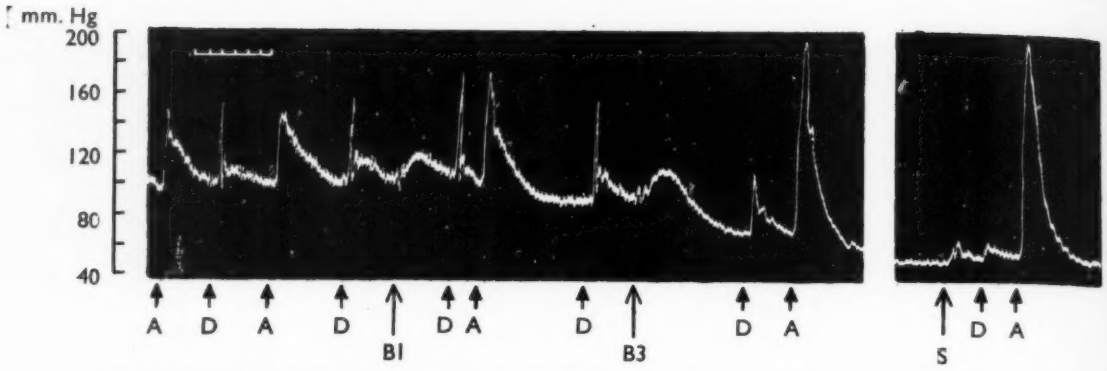


FIG. 12.—Record of the blood pressure of an adrenalectomized cat anaesthetized with chloralose. Adrenaline (10 μg.) was injected intravenously at **A** and 100 μg. of dimethylphenylpiperazinium at **D**. Bretylium was injected intravenously, 1 mg./kg. at **B1** and 3 mg./kg. at **B3**. **S** represents an injection of saline. Time, 1 min.

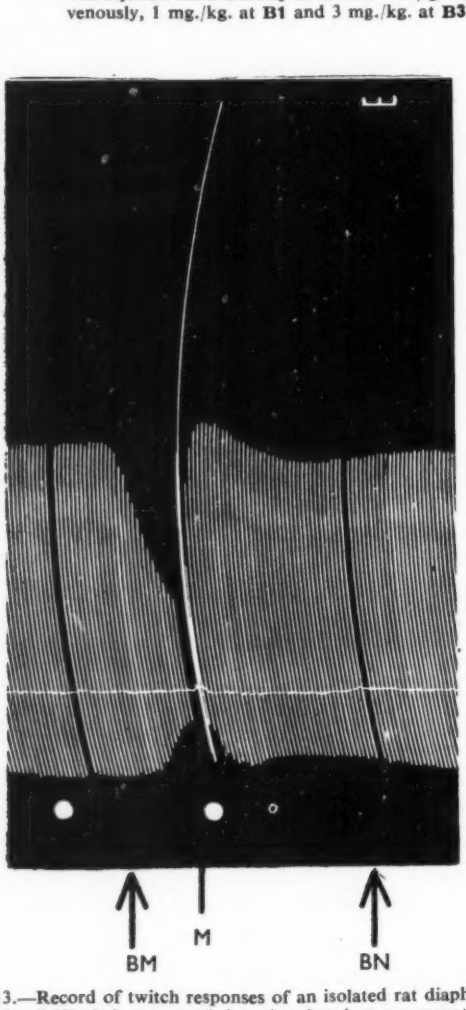


FIG. 13.—Record of twitch responses of an isolated rat diaphragm. Stimuli (5/min.) were applied to the phrenic nerve except at **M** which is a response to direct muscle stimulation. Bretylium was added to the organ bath to give 400 μg./ml. at **BM** and to the nerve only to give 10 mg./ml. at **BN**. The bath fluid was changed at white dots. Time, 1 min.

acetylcholine and 50 μg./ml. of choline 2,6-xylyl ether bromide. This action was inhibited by atropine (0.1 μg./ml.) and slightly reduced by hexamethonium (0.5 mg./ml.). A concentration of 100 μg./ml. did not reduce the sensitivity of the gut to acetylcholine (50 ng./ml.) or to 5-hydroxytryptamine (50 ng./ml.).

Neuromuscular Toxicity

Contractions of the isolated rat diaphragm in response to stimulation of the phrenic nerve were abolished by 400 μg./ml. of bretylium in the organ bath, whereas the sensitivity to direct muscle stimulation remained unchanged (Fig. 13). It was not antagonized by neostigmine. Topical application of 10 mg./ml. of bretylium to the phrenic nerve trunk did not depress conduction. Therefore the block seemed to be at or near to the neuromuscular junction.

In cats anaesthetized with chloralose, respiratory paralysis and inhibition of the response of the gastrocnemius muscle to indirect stimulation, but not to direct stimulation, occurred concomitantly after intravenous doses of 15 to 30 mg./kg. of bretylium. Thus, neuromuscular block seemed to be the main toxic effect of the drug. Subcutaneous doses of 100 mg./kg. caused muscular weakness in unanaesthetized cats but 50 mg./kg. was well tolerated. In monkeys, ptosis and a diminution in muscular effort often occurred after 50 mg./kg. In albino mice the LD50 of bretylium was approximately 16 mg./kg. intravenously, 68 mg./kg. subcutaneously and 270 mg./kg. when the drug was given by stomach tube. In two chicks 25 mg./kg. of bretylium intravenously caused a flaccid paralysis resembling that with curare. A dose of 37.5 mg./kg. intravenously was toxic in two other chicks.

Sensory Nerves

Local anaesthesia followed the intracutaneous injection of bretylium in concentrations of 5 mg./ml. or more in guinea-pigs. The effect was slow in onset but very prolonged even compared with that caused by cinchocaine (Fig. 14). Injection of a 30 mg./ml. solution left the site of injection not fully sensitive to stimulation after 48 hr. Similar observations were made using concentrations of 2.5 mg./ml. in man, a small area at the injection site remaining insensitive for over 24 hr.

Central Nervous System

No important behavioural change was observed in cats injected with amounts sufficient to relax the nictitating membrane, though lethargy was sometimes apparent after 10 to 30 mg./kg. of bretylium subcutaneously. No change in the activity counts of mice was detected at 15 to 25 min. after 50 mg./kg. of bretylium intraperitoneally nor at 2 hr. after 2.5 to 40 mg./kg. of bretylium tosylate intraperitoneally. However, at 4 and 6 hr. after 10 or 20 mg./kg. of bretylium tosylate, the mean activity counts were only about half those of the control mice and these differences were highly significant. The reduction in activity with 40 mg./kg. tended to be less than with 10 or 20 mg./kg. The duration of hypnosis following intravenous pentobarbitone sodium (60 mg./kg.) was slightly prolonged by the intravenous injection of 12.5 mg./kg. of bretylium at the same time, but not by 6.25 mg./kg. These effects could be due to peripheral changes.

In rats, no analgesic action was detected up to 6 hr. after 50 mg./kg. bretylium subcutaneously. The intracisternal injection of 0.5 mg. in 0.05 ml. of saline caused severe tremors for about 1 hr. The pupil diameters were unchanged.

Other Effects

Alimentary Tract.—No salivation or gastro-intestinal disturbance was apparent in cats. Salivation occurred after 10 to 30 mg./kg. of bretylium intravenously in the dog, and faecal evacuation after 30 to 100 mg./kg. subcutaneously in the rabbit and guinea-pig. Whether these effects should be attributed to parasympathomimetic action requires further investigation. The salivation in the dog could be due to the early sympathomimetic effect of the drug, and the faecal evacuation in the other two species could be consequent to a diminution in sympathetic tone. The proportion of the small intestine traversed by a charcoal meal administered to fasted rats was not materially changed by the previous administration of bretylium. In an experiment with groups of six rats the mean percentage of the small intestine traversed in 10 min. was 34 in controls given saline. In the groups given 10 and 40 mg./kg. of bretylium 1 hr. before the charcoal the values were 34 and 32% respectively. When 10 and 40 mg./kg. were given 6 hr. before the meal the values were 45 and 41%. The differences are not statistically significant, though bretylium might have accelerated gastro-intestinal propulsion. The peristaltic reflex elicited by distending the isolated guinea-pig ileum was abolished by 30 μg./ml. bretylium.

Isolated Heart Preparations.—In each of three Langendorff preparations of the rabbit heart, the injection of 0.05 to 0.5 mg. of bretylium into the arterial cannula caused small transient reductions in the coronary flow and the strength of the ventricular contractions. No change in heart rate was detected. Strips of guinea-pig ventricles, driven at the rate of two contractions/min. by direct muscle stimulation (Stewart, 1958), showed a small positive inotropic response with bretylium (10 to 20 μg./ml.).

Uterus.—No change in the tone of isolated guinea-pig or rabbit uterus was observed until the concentration of bretylium was increased to between 100 and 300 μg./ml., at which a small contraction occurred. Rat uterus showed no response to this concentration, but its sensitivity to

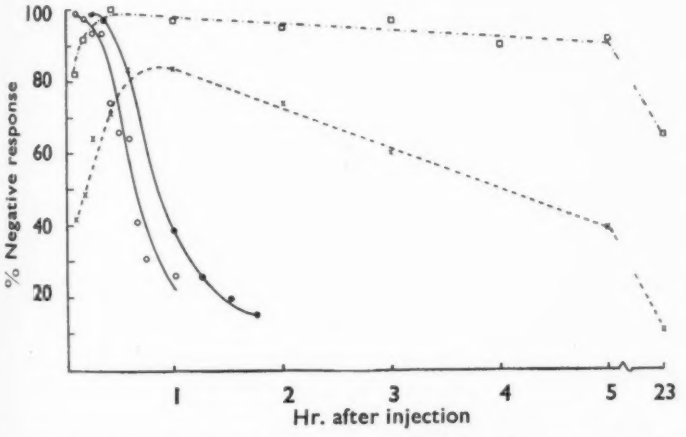


FIG. 14.—Local anaesthesia after the intradermal injection of bretylium and cinchocaine hydrochloride in guinea-pigs. Ordinate: the % of negative responses to six pricks on four wheals in each of three guinea-pigs (total of 72 responses). × and □, Bretylium 10 and 30 mg./ml. respectively; O and ●, Cinchocaine hydrochloride 0.5 and 1.5 mg./ml. respectively.

5-hydroxytryptamine (0.1 μg./ml.) was impaired.

Histamine.—No antihistamine effect on the blood pressure was detected in cats after the injection of 10 mg./kg. of bretylium intravenously, but concentrations between 1 and 10 μg./ml. reduced the submaximal contractions of the isolated guinea-pig ileum preparation stimulated by histamine.

Bretylium Tosylate

The tosylate (*p*-toluenesulphonate) is preferred to the bromide for clinical use and was chosen because it was the least hygroscopic of the many alternative salts examined. The anion did not appear to influence the pharmacological action of the bretylium cation. The toxicity of bretylium tosylate was equivalent on a molar basis to that of the bromide in comparative tests. The LD50 of the bretylium tosylate in mice was approximately 20 mg./kg. by intravenous injection, 72 mg./kg. subcutaneously and 400 mg./kg. orally. The tosylate anion was not expected to contribute to the toxicity of the bretylium salt, since in mice the intravenous LD50 of sodium paratoluene sulphonate is 1,700 mg./kg. Comparison of bretylium tosylate and bretylium bromide in cats showed that they had equivalent effects on blood pressure and on the nictitating membrane and that their toxicities were equivalent. Amounts of up to 400 mg./kg. of bretylium tosylate were tolerated when given by stomach tube to rats, as was 200 mg./kg. by the same route in the monkey.

Discussion

Bretylium specifically depressed the peripheral sympathetic nervous system. Since the responses to stimulation of several different postganglionic sympathetic nerves were depressed as readily as responses to preganglionic stimulation, the depressant action is peripheral to the ganglia. While bretylium depressed the end-organ responses to stimulation of all the postganglionic adrenergic nerves of the sympathetic nervous system investigated, it did not depress the function of the postganglionic cholinergic nerves of this system. Thus the production of sweat from the paw of the cat during stimulation of its postganglionic nerve supply continued after administration of the drug. Further, after bretylium had been given in sufficient amounts to prevent the tachycardia caused by stimulation of the cardioaccelerans nerve, stimulation of the latter caused a bradycardia which was resistant to further doses of bretylium but abolished by atropine. The parasympathetic nervous system was unaffected by amounts of bretylium sufficient to depress the sympathetic, but large intravenous doses caused signs of a block in synaptic transmission in parasympathetic and sympathetic ganglia. These effects were brief and occurred only with doses which impaired neuromuscular transmission. No antimuscarine action was detected.

The block of responses mediated by adrenergic nerves was not due to an antagonism of adrenaline or noradrenaline. The effects of these compounds were enhanced after giving bretylium, as they are after sympathectomy. Experiments on the spleen have shown that its adrenergic nerve failed to release noradrenaline after bretylium. This failure might be attributable to an impairment of transmission along the neurone since bretylium could block adrenergic nerve trunks when applied topically. As mentioned in preliminary reports (Boura *et al.*, 1959a and b), bretylium administered systemically does accumulate selectively in adrenergic nerves in sufficient concentrations to impair conduction (Boura, Copp, Duncombe, Green and McCoubrey, unpublished observations). That the finer terminal nerve endings may be more sensitive or accessible than the nerve trunks to the drug is suggested by experiments on the isolated rabbit intestine and uterus and on the perfused rabbit ear. Higher concentrations were needed to cause a local block of nerve trunks than were needed to depress the end-organ response when the bretylium was applied to the whole preparation and consequently gained access to the nerve endings. The cell body of the adrenergic neurone might be expected to be sensitive to bretylium, though experiments in which the drug was given by close arterial injection or into the fluid perfusing the isolated ganglion do not differentiate between effects at ganglionic synapses and effects on adrenergic neurones.

Bretylium also depressed transmission in other nerves when applied locally. Local anaesthesia of long duration was produced by the intracutaneous injection of the drug in guinea-pig and man, though the concentrations needed were higher than those which depressed adrenergic nerves. This correlates with the observation that many local anaesthetics depress conduction in adrenergic nerves (such as vasoconstrictor fibres) in lower concentrations than are needed to depress sensory conduction (Gaddum, 1953). The phrenic nerve of the rat was insensitive to the local application of high concentrations of bretylium, perhaps because the myelin sheath prevents the penetration of the bretylium cation.

The pressor response to stimulating the adrenal medulla, either by splanchnic nerve stimulation or by the intravenous injection of dimethylphenylpiperazinium, was increased rather than impaired by bretylium and this is a further contrast between bretylium and ganglionic blocking agents. The increased response to stimulation of the medulla may be accounted for by an enhancement of the effect of the released adrenaline and noradrenaline. The contrast between the effect of the drug on the adrenal medulla and the effect on adrenergic neurones is in keeping with the contention that the drug affects nerve conduction and not the reactivity of chromaffin tissue in general. In this connexion, it is of interest that, while bretylium did not inhibit the action of dimethylphenylpiperazininum on the adrenal medulla, it antagonized the pressor effects of larger doses of this drug in the adrenalectomized cat and likewise the pressor effect of acetylcholine in the atropinized adrenalectomized cat.

Many of the effects of bretylium on sympathetic function resemble those of choline 2,6-xylyl ether bromide (Hey and Willey, 1954; Exley, 1957; Edge, Mason, and Wyllie, 1957) but, in contrast, bretylium even in near toxic concentrations did not cause parasympathomimetic effects *in vivo*. However, high concentrations of bretylium (200 µg./ml.) might be regarded as muscarine-like in action, for they cause a spasm in isolated strips of guinea-pig ileum which was affected but little by hexamethonium but was antagonized by atropine. This action is less than that of choline 2,6-xylyl ether bromide.

An inhibition of conduction in adrenergic neurones was thought by Hey and Willey (1954) to explain the depressant effects of choline 2,6-xylyl ether bromide on the peripheral sympathetic system. They showed that this drug had a powerful long-lasting local anaesthetic effect, and that injected procaine would inhibit the effect of stimulating the postganglionic cervical sympathetic nerves. However, the apparent failure of the drug to impair action potentials on adrenergic nerves (Exley, 1957), the reductions in the catechol amine content of the rat adrenal medulla found after its administration (Coupland and Exley, 1957; Coupland, 1958) and a reduction in the synthesis of noradrenaline by phaeochromocytoma tissue (Bain and Fielden, 1957) led to the suggestion that choline 2,6-xylyl ether bromide acted by inhibiting the synthesis of noradrenaline in adrenergic nerves. Bretylium does not seem to act specifically in this way (Boura, Green, and McCoubrey, unpublished observations). Its mechanism of action seems to be similar to that first postulated for choline 2,6-xylyl ether bromide by Hey and Willey (1954).

The reduction caused by bretylium in the regression of the effect on the nictitating membrane of the frequency of stimulation applied to its adrenergic nerve supply (Fig. 5) may be of considerable significance. At the low rates of stimulation a small increase in the stimulation rate compensated for the administration of a threshold dose of bretylium (2 to 6 mg./kg.); but at the higher rates a large increase, often beyond normal physiological limits, was required. This situation may allow functional specificity of action on different adrenergic nerves, the impairment being greatest in those subjected to the greatest traffic of impulses. It could be a factor in postural hypotension, the frequency of sympathetic impulses being higher when the animal is tilted towards an erect position. It suggests that supine hypertensive patients show a greater fall in the blood pressure after bretylium than do the normotensive individuals (Boura *et al.*, 1959b) because their sympathetic tone is greater. Similar arguments have been used in relation to the action of the ganglionic blocking agents since, under similar circumstances, they block more readily when the frequency of stimulation is high (Paton, 1951; Green, 1956), but, however, the analogy between the two drugs ends here. Ganglionic blocking agents antagonize acetylcholine competitively at the ganglionic synapses and have a greater effect on high rates of stimulation applied continuously because the acetylcholine released at the ganglion falls during the period of stimulation. Because of this the nictitating membrane of a cat which has received a ganglionic blocking agent may show a characteristic spike contraction during a period of continuous tetanic stimulation of the preganglionic nerve. After bretylium has been given to cats in which the membrane is stimulated through its postganglionic nerve, such spike contractions do not occur. The membrane contraction may be greatly reduced but is fairly well sustained during continuous stimulation.

Cocaine antagonized the effect of bretylium, blocking adrenergic nerves to the isolated rabbit gut and the nictitating membrane of the cat, in doses which increased the sensitivity to adrenaline, noradrenaline, and nerve stimulation. Nasmyth and Andrews (1959) have shown that cocaine antagonizes the adrenergic nerve block caused by choline 2,6-xylyl ether bromide.

Sympathomimetic effects were seen after the administration of large doses of bretylium. They followed rapidly after intravenous injection into

the cat, were brief on the blood pressure and heart rate, but last longer on the nictitating membrane. They occurred in adrenalectomized animals and those with spinal transection. The effect on the nictitating membrane persisted after cutting the postganglionic nerve and could be produced by intra-arterial injection either into the region of the ganglion or into the internal carotid artery. Vasoconstriction occurred after large amounts were injected into the perfused rabbit ear. It is therefore concluded that the stimulatory actions, like the inhibitory effects of bretylium on the sympathetic nervous system, may be due to an action of the drug on the adrenergic neurones.

High concentrations of bretylium caused a curare-like paralysis, and this was the main toxic effect of the drug. In the chick, bretylium caused flaccid paralysis, similar to that which Buttle and Zaimis (1949) found distinguished the action of curare from that of depolarizing drugs. On the other hand, the neuromuscular paralysis produced in the rat diaphragm preparation, in contrast to that caused by curare, was not antagonized by neostigmine.

When bretylium was given orally, the adrenergic neurone blocking action in the cat and the toxic effects in the cat, mouse, and rat occurred only after three to five times the doses which produced these effects by the subcutaneous route. This indicates that only about a third of the dose may be absorbed, a not uncommon proportion with a quaternary ammonium salt. This does not, however, introduce the complication encountered in the clinical use of ganglionic blocking agents. In contrast to them, bretylium lacked the parasympathetic blocking effect of delaying intestinal transport which may result in the accumulation of unabsorbed drug in the intestine and consequent paralytic ileus. No untoward disturbance of gastrointestinal function was found after administration of bretylium in man (Boura *et al.*, 1959b).

We are indebted to many colleagues at these Laboratories and especially to Dr. A. C. White and the following assistants in the department: Miss D. R. Billinghurst, Mrs. E. E. Diprose, Mr. F. Huggins, Miss C. Kingswell, Mrs. I. A. Saunders, and Mr. T. D. Whiting. The experiments on the perfused ganglion were carried out by Mrs. Naomi B. Higson.

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KININS PRODUCED FROM BOVINE COLOSTRUM BY KALLIKREIN AND SALIVA

BY

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Substances capable of stimulating smooth muscle are produced on the incubation of bovine colostrum with urinary kallikrein or calf saliva. These substances, called urine- and saliva-colostrokinin, have been differentiated from kallidin, substance A and similar smooth muscle activating agents. Saliva-colostrokinin is likely to be formed in the suckling calf. Further, as colostrum became milk, the ability to form colostrokinin diminished. A function for saliva-colostrokinin in the newborn is suggested.

Kallikrein is a substance occurring in the urine, pancreatic juice, salivary glands, and saliva of many animals (Frey, Kraut, and Werle, 1950). Its properties indicate that it is an enzyme or mixture of enzymes similar to but not identical with trypsin (Frey *et al.*, 1950 ; Werle, 1955 ; Berg and Beeler, 1955). Kallikrein causes a fall in blood pressure and an inhibition of induced spasticity in the stomach, bronchi, and heart. Selected smooth muscle preparations are stimulated by kallikrein, but most of the more commonly employed smooth muscle organs remain unaffected (Frey *et al.*, 1950 ; Werle, 1955).

When kallikrein is incubated with whole plasma or with suitable plasma protein fractions such as the α_2 globulins or Cohn fraction IV-4, smooth muscle stimulating activity is developed. The substance causing this activity has been called kallidin (Frey *et al.*, 1950 ; Werle, 1955).

Evidence has accumulated indicating that kallidin belongs to the group of polypeptides called the kinins, such as wasp kinin, bradykinin, glass-activated kinin, and urinary kinin (Werle, 1953 ; Gaddum and Horton, 1959 ; Holdstock, Mathias and Schachter, 1957). In accordance with recent practice (Gaddum and Horton, 1959 ; Horton, 1958 ; Horton, 1959 ; Margolis, 1958 ; Lewis, 1958 ; Hilton and Lewis, 1958) any smooth muscle stimulating substance produced by the action of kallikrein on plasma protein will be called a plasma kinin.

Colostrum proteins appear to be derived from the proteins of plasma (Crowther and Raistrick, 1916 ; Askonas, Campbell, Humphrey, and Work, 1954 ; Larson and Gillespie, 1957). When kallikrein is incubated with colostrum a smooth muscle stimulating substance is produced (Werle, 1959 ; Guth, 1959). This substance has been called colostrokinin by Werle (1959). It will be called urine-colostrokinin in this report, to indicate the source of the kallikrein and to differentiate it from another kinin formed from colostrum protein.

Saliva or saliva constituents are capable of producing plasma kinins on incubation with plasma protein (Frey *et al.*, 1950 ; Hilton and Lewis, 1957). Saliva is physiologically incubated with colostrum by the newborn in the act of suckling. The kinin formed by the reaction of saliva and colostrum will be called saliva-colostrokinin. The formation of kinins by the reaction of colostrum and saliva is likely to be a common physiological occurrence. It is conceivable that the kinin formed in the saliva-colostrum mixture subserves some physiological function in the newborn.

METHODS

Rat Uterus.—The uteri from virgin rats (150 to 250 g.) were suspended in De Jalon solution in a 3 ml. bath at 30°.

Guinea-pig Ileum.—The terminal segments of ileum from guinea-pigs weighing 180 to 300 g. were suspended in Tyrode solution in a 3 ml. bath at 37°. Atropine sulphate (1 mg./l.) was added when spontaneous movements were not otherwise controllable.

Rat Duodenum.—The proximal segments of the duodenum from rats weighing 150 to 250 g. were suspended in De Jalon solution in a 3 ml. bath at

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30 to 37°. The tissue was stored at 4° for 2 to 3 hr. before use.

The dose cycle was usually repeated at 3 min. intervals. Using a slow continuous flow of fluid from below, the preparations seemed most responsive after a 30 sec. equilibration period during which the inflow was stopped. The substances under investigation were injected at the end of this equilibration period. The inflow was started 15 sec. after injection with the duodenum and 1 min. after injection with the ileum and uterus preparations.

Preparation of Human and Sheep Urinary Kallikreins.—Urine from men and Clun Forest wether sheep was put under toluene and frozen until used. The procedure for preparing kallikrein was that described by Gaddum and Horton (1959), with the exception that the urine was concentrated by placing it in dialysis bags and hanging them before a fan and heat source rather than by using a climbing film evaporator.

Plasma Kinin.—Human urinary kallikrein (1 mg./10 ml. mixture) was allowed to stand with acid-treated dialysed dog plasma for 2 hr. at room temperature. The mixture was then poured into twice its volume of boiling absolute alcohol. When boiling resumed, it was allowed to continue for 5 min. The supernatant fluid and the precipitate were separated by centrifugation for 15 min. at 2,500 revs./min. The precipitate was washed once by centrifugation with 66% (v/v) ethanol. The supernatant fluids were combined and their volume reduced by vacuum distillation at 50 to 60°. The final evaporation was performed in a lyophilizer. The dried mass was powdered in a mortar and passed through a No. 60 sieve.

Urine-colostrokinin. — Sheep urinary kallikrein (1 mg./10 ml. mixture) was allowed to stand with acid-treated dialysed bovine colostrum for 2 hr. at room temperature. The procedure for the preparation of plasma kinin was then employed.

Saliva-colostrokinin.—Dialysed calf saliva (1 ml./10 ml. mixture) was allowed to stand with acid-treated dialysed bovine colostrum for 2 hr. at room temperature. The procedure for the preparation of plasma kinin was then employed.

Acid treatment of plasma was found by Horton (1958) to inhibit kallikreinase and kininase without affecting kinin formation. Preliminary experiments indicated that acid treatment might not be necessary with colostrum. It was nevertheless used to make the kinins of plasma and colostrum more strictly comparable. The kinin produced by reaction of sheep urinary kallikrein and colostrum is called urine-colostrokinin. That produced by reaction of saliva and colostrum is called saliva-colostrokinin.

The saliva used in these experiments was always obtained from the calf whose dam kindly donated the colostrum.

Kallikrein Substrate Content of Colostrum.—The procedure outlined for the preparation of kinins was designed to promote maximum kinin formation. Excess kallikrein was used and the incubation period was about twice as long as necessary for the reaction to go to completion. The limiting factor of the reaction was therefore the concentration of substrate in the colostrum. The relative content of kallikrein substrate was estimated by assaying the kinins so produced against a standard plasma kinin.

Enzyme Destruction Studies.—Two different trypsin preparations were used (Armour and Co. and Light and Co.). The trypsin was incubated at a concentration of 100 μg./ml. with solutions of plasma kinin, urine-colostrokinin, and saliva-colostrokinin containing 1 mg./ml. Samples were taken and tested on the isolated rat uterus. Chymotrypsin (Sigma Chemical Corp.) was tested in the same manner. Appropriate controls were employed.

RESULTS

The production of a smooth muscle stimulating substance (saliva-colostrokinin) when dialysed calf saliva was incubated with acid-treated

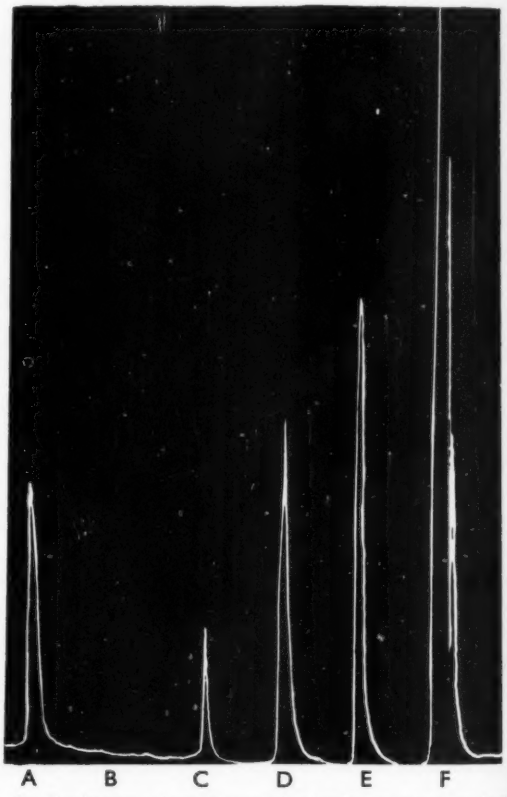

FIG. 1.—Production of smooth muscle stimulating substance (saliva-colostrokinin) in a mixture of saliva and colostrum. The isolated rat uterus in De Jalon solution is the test organ. At **A**, 100 μg. of standard plasma kinin was injected into bath; **B**, 0.2 ml. of dialysed colostrum was added; **C**, 0.1 ml. calf saliva. After C, 3 ml. calf saliva was mixed with 22 ml. colostrum. **D**, **E**, and **F** are the effects of 0.1 ml. of the mixture taken 1, 4, and 7 min. after mixing. Bath vol., 3 ml.

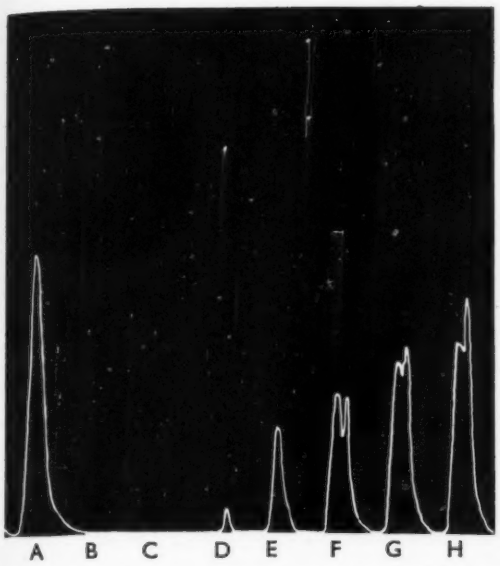

FIG. 2.—Production of smooth muscle stimulating substance (urine-colostrokinin) in a mixture of kallikrein and colostrum. The isolated rat uterus in De Jalon solution is the test organ. At A, 100 μg. of standard plasma kinin is injected into the bath; B, 0.2 ml. dialysed bovine colostrum; C, 0.1 ml. sheep urinary kallikrein (5 μg./ml.). After C, 5 ml. of sheep urinary kallikrein solution (1 mg./ml.) was added to 10 ml. of dialysed acid-treated bovine colostrum. D to H are the effects of 0.1 ml. of incubation mixture taken every 3 min.

dialysed bovine colostrum is shown in Fig. 1. Fig. 2 illustrates the production of a smooth muscle stimulating substance (urine-colostrokinin) when sheep urinary kallikinin was incubated with acid-treated dialysed bovine colostrum.

The experiments in which plasma kinin, urine-colostrokinin, and saliva-colostrokinin were incubated with proteases showed that chymotrypsin, as had been expected, destroyed the activities of all three kinins. The Armour trypsin preparation destroyed none of them. As the trypsin preparation of Light and Co. destroyed the activity of plasma kinin without affecting that of urine-colostrokinin or saliva-colostrokinin, it is suggested that there may be a basic difference between plasma kinin and the colostrokinins.

TABLE I

PARALLEL ASSAYS OF PLASMA KININ, URINE-COLOSTROKININ, AND SALIVA-COLOSTROKININ

Milligram doses equiactive with 1 mg. of plasma kinin on three smooth muscle preparations are given. These values are the means of at least four six-point assays. The numerals in parentheses indicate the range. I.D. is the Index of Discrimination obtained by dividing the value for one organ by that obtained on a different organ. An I.D. near unity indicates no detectable differences between the substances being assayed.

	Saliva-colostrokinin	No. of Assays	Urine-colostrokinin	No. of Assays
Rat uterus ..	44 (25–50)	6	6·9 (6·6–7·5)	4
,, duodenum ..	16·5 (10–25)	4	6·0 (4·0–8·0)	4
Guinea-pig ileum	8·1 (7·7–10·0)	5	4·5 (3·3–6·0)	4
I.D. (rat uterus/ rat duodenum)	2·7		1·1	
I.D. (rat uterus/ guinea-pig ileum)	5·4		1·5	

Table I summarizes the results of parallel assays. The index of discrimination (Gaddum, 1955) provides a measure of the dissimilarity between possibly-related substances. An index near unity indicates that the test preparations used are incapable of discriminating between the substances under test. These results show that plasma kinin and urine-colostrokinin are more nearly identical than are plasma kinin and saliva-colostrokinin. The differences between plasma kinin and saliva-colostrokinin are, however, relatively small and may be reconciled when purer preparations of the kinins become available.

From Fig. 3 it may be seen that, as bovine colostrum becomes milk, the kallikrein substrate concentration diminished. This diminution coincided in the calf with the time at which the permeability of the gut to proteins diminished (Deutsch and Smith, 1957).

Substance A (Huggins and Walaszek, 1959) is produced by incubating plasma protein fraction IV-4 with salivary α-amylase. It might seem a good candidate for comparison with saliva-colostrokinin. Three considerations militate against the identity of substance A and saliva-colostrokinin: substance A is destroyed by trypsin while saliva-colostrokinin is not (Walaszek and

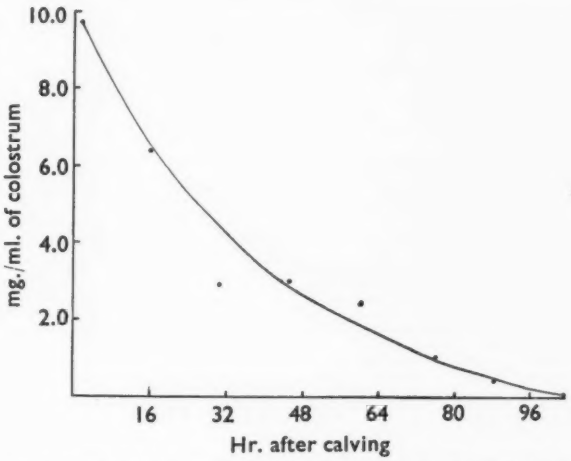

FIG. 3.—Diminution in kallikrein-substrate as bovine colostrum becomes milk. Samples of bovine colostrum taken at various intervals after calving were incubated with sheep urinary kallikrein. The urine-colostrokinin produced was assayed against plasma kinin, and the relative activities produced expressed as mg. equivalents of plasma kinin are plotted against time.

Huggins, 1959); the content of α-amylase in calf saliva is very low (Matsuoka, 1937); and an indirect comparison between substance A and the colostrokinins on the isolated rat duodenum suggested they were different. The rat duodenum preparation often responded to substance A in a biphasic manner. The colostrokinins were pure relaxants.

Discussion

The relaxation of the isolated rat duodenum by the colostrokinins permits them to be distinguished from substance P, acetylcholine, histamine, angiotensin, and 5-hydroxytryptamine. Adenosine and adenosine monophosphate both relax the isolated rat duodenum, but never to the same extent as the kinins (Horton, 1959). The doses of vasopressin and oxytocin that relax the rat duodenum are at least one hundred times greater than those necessary for contraction of the isolated rat uterus (Horton, 1959). The kinins are about equipotent on the two preparations. On this single preparation, the isolated rat duodenum, the kinins may be distinguished from many smooth muscle activating agents.

Kallikrein is probably the prime agent in saliva responsible for the production of saliva-colostrokinin. Hilton and Lewis (1957) and Holdstock *et al.* (1957) have used whole saliva or saliva extracts for the activation of plasma proteins. Werle (1955) and Frey *et al.* (1950) have demonstrated the presence of kallikrein in saliva. They have also shown that the kallikreins of saliva and urine are different (Frey *et al.*, 1950; Werle, 1955). The protein substrates of colostrum and plasma are probably not identical. Urinary kallikrein acting on plasma and on colostrum produces two kinins, one of which is destroyed by a single trypsin preparation. Considering that the substrates in colostrum and plasma may be different and that the kallikrein of urine and saliva are different, it is not surprising that small differences appeared in parallel assay experiments. It is too early to be certain that saliva-colostrokinin and urine-colostrokinin are identical.

Kinins are capable of increasing capillary permeability (Holdstock *et al.*, 1957). By this property saliva-colostrokinin might aid in the transport of proteins across the neonatal gut to the blood, but further work on this aspect is needed.

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Brit. J. Pharmacol. (1959), **14**, 553.

THE USE OF THE GUINEA-PIG ILEUM PREPARATION FOR TESTING THE ACTIVITY OF SUBSTANCES WHICH IMITATE OR ANTAGONIZE THE ACTIONS OF 5-HYDROXYTRYPTAMINE AND TRYPTAMINE

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On the guinea-pig ileum, 5-hydroxytryptamine appeared to act in the same way as tryptamine on two types of receptor (morphine-sensitive and phenoxybenzamine(Dibenzyline)-sensitive). The actions of analogues of 5-hydroxytryptamine on the phenoxybenzamine-sensitive receptors resembled their actions on the rat uterus, and the actions on the morphine-sensitive receptors slightly resembled those on the rat fundus strip. The guinea-pig ileum preparation, however, did not appear to be more suitable than the rat uterus and rat fundus strip for testing the ability of compounds to imitate or antagonize 5-hydroxytryptamine.

Analogues of 5-hydroxytryptamine are of interest, for not only may they imitate or antagonize the effects of 5-hydroxytryptamine on peripheral tissues but they may have profound effects on the central nervous system. It is difficult to test great numbers of compounds on the central nervous system itself and it is, therefore, desirable that the peripheral tissues chosen for the initial testing of activity should be those on which this activity is most likely to run parallel with that on the central nervous system.

Costa (1956) reported that tranquillizers, such as azacyclonol (Frenquel), chlorpromazine, and reserpine, antagonized the actions of 5-hydroxytryptamine on the rat uterus whereas hallucinogens, such as mescaline and lysergic acid diethylamide, increased the sensitivity to 5-hydroxytryptamine. Cerletti and Doepfner (1958), however, could not obtain any such facilitation with hallucinogens, and it is doubtful whether results obtained on this preparation yield much information about the possible effects of compounds on the central nervous system.

This paper describes experiments with analogues of 5-hydroxytryptamine on the guinea-pig ileum. This tissue contains two types of receptor sensitive to 5-hydroxytryptamine, referred to as M (morphine-sensitive) and D (phenoxybenzamine(Dibenzyline)-sensitive) by Gaddum and Picarelli (1957). The activity of compounds on the M receptors, which are thought to be located in nervous tissue, was considered more likely to run parallel with that on the central nervous system than was the activity on any other preparation in use at the time (J. H. Gaddum, personal communication).

Gaddum (1953) had shown that, after large doses of either 5-hydroxytryptamine or tryptamine, the guinea-pig ileum became insensitive to both 5-hydroxytryptamine and tryptamine. Because of this Gaddum and Picarelli (1957) assumed that the two drugs acted on the same receptors in the guinea-pig ileum. They referred to the M and D receptors as tryptamine receptors even though their experiments were performed with 5-hydroxytryptamine. Woolley and Shaw (1957) and Barlow and Khan (1959a and b) have found substances which antagonize 5-hydroxytryptamine more than tryptamine, which suggests that there may be separate tryptamine and 5-hydroxytryptamine receptors. It was, therefore, desirable to confirm first that the results obtained with 5-hydroxytryptamine on the guinea-pig ileum could also be obtained with tryptamine. This was found to be true and so a number of analogues have been tested on both the M and the D receptors of the guinea-pig ileum. The results are compared with those obtained on the rat uterus and the rat fundus strip (Barlow and Khan, 1959a and b),

and an attempt has been made to assess the value of the estimates of activity obtained with each preparation.

METHODS

The Isolated Guinea-pig Ileum Preparation.—Terminal pieces of ileum from guinea-pigs (150 to 200 g.) deprived of food overnight were suspended in Tyrode solution at 35° in a 2 ml. bath, through which air was bubbled. The movements of the gut were recorded with a light frontal-writing lever, with 5 × magnification, on a smoked paper.

Comparison of 5-Hydroxytryptamine and Tryptamine. — To see if 5-hydroxytryptamine and tryptamine were acting in the same way on the guinea-pig ileum, the doses for equivalent activity of each drug were determined before and after treatment with morphine (3.5×10^{-6} M), phenoxybenzamine (3.4×10^{-7} M) and lysergic acid diethylamide (3×10^{-8} M); the results are expressed as dose ratios. The concentrations of these antagonists were identical with those used by Gaddum and Picarelli (1957). Acetylcholine and nicotine were used as control drugs. Although it was not necessary in determining the dose ratios to plot the complete dose/response curves for 5-hydroxytryptamine and tryptamine, this was done for both drugs before and after the action of lysergic acid diethylamide and for 5-hydroxytryptamine only before and after the action of phenoxybenzamine.

Investigation of the Analogues.—The compounds used are listed in Table IV. Some (see acknowledgments) were given to us; the others we synthesized (Barlow and Khan, 1959a and b).

Exactly the same procedure was used as in the experiments on the rat uterus and rat fundus strip (Barlow and Khan, 1959a), except that the Tyrode solution contained either 3.5×10^{-6} M morphine or 3×10^{-8} M lysergic acid diethylamide.

Experiments were also done on preparations which had been treated with 3.4×10^{-7} M phenoxybenzamine for 30 min. and then washed and suspended in normal Tyrode solution. After this treatment, reproducible responses could only be obtained with 5-hydroxytryptamine for about 2 hr. Thus in any one experiment it was only possible to compare one compound with 5-hydroxytryptamine. In the presence of morphine and lysergic acid diethylamide, however, it was possible to compare 5-hydroxytryptamine with more than one analogue.

RESULTS

5-Hydroxytryptamine and Tryptamine

Dose Ratios.—The dose ratios of 5-hydroxytryptamine and tryptamine for morphine, phenoxybenzamine, and lysergic acid diethylamide are shown in Table I. The values for 5-hydroxytryptamine and tryptamine agree reasonably well, justifying the use of the term tryptamine receptors by Gaddum and Picarelli (1957).

Phenoxybenzamine-treated tissue was so insensitive to the action of tryptamine that no attempt was made to obtain dose ratios with tryptamine. The dose ratios for phenoxybenzamine and lysergic acid diethylamide are given as ranges because, after the preparation had been treated with these drugs, the dose/response curves to 5-hydroxytryptamine and tryptamine, although parallel with each other, were not parallel with the original curves (Fig. 1). This may explain why our estimates of these dose ratios are higher than those (for 5-hydroxytryptamine) of Gaddum and Picarelli (1957). The doses of agonist producing a 50% response varied from one preparation to another depending upon individual sensitivity. Consequently the values of the dose ratios will not be consistent if the dose/response curves for the agonists in the presence of the

TABLE I

ANTAGONISTIC ACTIVITY OF LYSERGIC ACID DIETHYLAMIDE, PHENOXYBENZAMINE, AND MORPHINE ON THE GUINEA-PIG ILEUM AT 35°

The two limits of the ratios for phenoxybenzamine and lysergic acid diethylamide are taken from the two extremes of the dose/response curves of 5-hydroxytryptamine and tryptamine. — Indicates not tested. For morphine, means with standard errors are given.

Drug	No. of Expts.	Conc. ($\times 10^{-7}$ M)	Dose Ratio at the End of 1 hr. for			
			5-Hydroxytryptamine	Tryptamine	Nicotine	Acetylcholine
Phenoxybenzamine	5	3·4	80–40 90–80 100–10 100–20 100	— — — — —	1 1·5 2 3 2	— — — — —
Lysergic acid diethylamide	5	0·3	40–30 50–35 10–8 10–8 25–15	80–40 40–25 40–25 13 —	1 1 1 1 —	1 1 1 1 1
Morphine	4	35	3±0·5	3±0·5	3·5±0·4	1±0

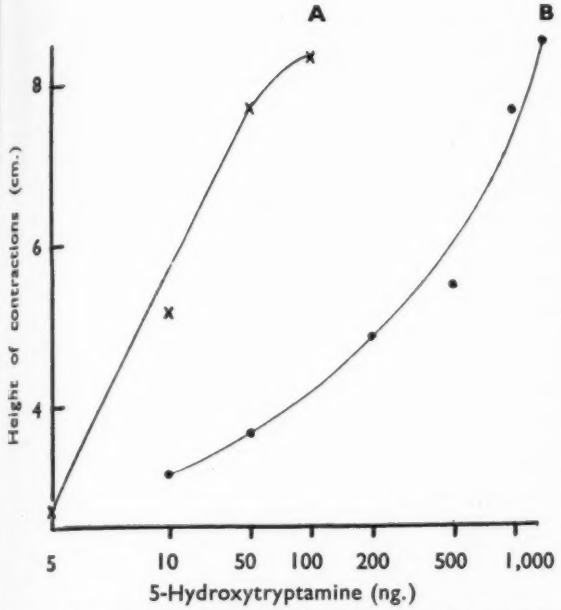


FIG. 1.—Isolated guinea-pig ileum preparation. Log dose/response curves with 5-hydroxytryptamine before (A) and after (B) the action of lysergic acid diethylamide 3×10^{-8} M. Temperature 35°.

antagonists are not parallel with those for the agonists alone.

Effects of Increasing the Concentration of Morphine and Lysergic Acid Diethylamide.—Kosterlitz and Robinson (1955, 1958) showed that increasing the concentration of morphine above 1.7×10^{-7} M did not increase its antagonism of 5-hydroxytryptamine (a concentration 470 times larger, 8×10^{-5} M, gave the same dose ratio), and Gaddum and Picarelli (1957) found that with 3.5×10^{-6} M morphine the M receptors appeared to be more or less completely blocked. Further, it has been shown by Gaddum and Hameed (1954) that raising the concentration of lysergic acid diethylamide from 3×10^{-8} M to 3×10^{-5} M did not increase the antagonism of 5-hydroxytryptamine.

These experiments have been repeated using tryptamine, nicotine, and acetylcholine as well as 5-hydroxytryptamine, and it was found that tryptamine behaved like 5-hydroxytryptamine. The concentration of morphine (3.5×10^{-6} M) produced a maximal antagonism of 5-hydroxytryptamine, tryptamine, and nicotine, but had no effect on responses to acetylcholine. A ten-fold rise in the concentration of morphine did not increase the antagonism of 5-hydroxytryptamine, tryptamine, and nicotine, and had no effect against acetylcholine.

The concentration of lysergic acid diethylamide used (3×10^{-8} M) produced maximum antagonism of 5-hydroxytryptamine and tryptamine, but had no effect against nicotine. This resistance of the effects of nicotine to antagonism by morphine confirms the findings of Kosterlitz and Robinson (1955, 1958).

Effect of Temperature.—In the first experiments with the guinea-pig ileum, the temperature was 35° and there was considerable spontaneous activity of the tissue. In order to reduce this, the temperature was lowered to 28°, and it was then found that the effects of morphine were very feeble. The dose ratios using 3.5×10^{-6} M

TABLE II

ANTAGONISTIC ACTIVITY OF SOME INDOLE DERIVATIVES ON THE M AND D RECEPTORS IN THE GUINEA-PIG ILEUM AT 35°

5-HT, 5-hydroxytryptamine; N, nicotine; H, histamine; Ach, acetylcholine, and — = not tested. Antagonism of 5-hydroxytryptamine by *N'N'*-dimethyltryptamine and 5-benzyloxy-*N'N'*-dimethyltryptamine was maximal at the end of 1 hr., but their antagonism of histamine was maximal within 15 to 20 min. Antagonism of nicotine by 5-benzyloxy-*N'N'*-dimethyltryptamine was maximal only after at least 1 hr. On the phenoxybenzamine-treated preparation the antagonists were only allowed to act for 15 min., but no effects were observed.

Serial No.	Drug	Conc. ($\times 10^{-7}$ M)	Dose Ratios at the End of 1 hr.						
			In Presence of Morphine (D Receptor)					After Phenoxybenzamine Treatment (M Receptor)	
			No. of Expts.	5-HT	N	H	Ach	No. of Expts.	5-HT
8	5-Benzyloxygramine	3·6	2	14 9	1 1	1 1	— —	1	1 —
		7·2	1	15	3	1	—		—
7	5-Benzyloxy-*N'N'*-dimethyltryptamine	62	3	33 36 110	130 100 66	20 3 7	1 1 1	1	1 — —
6	*N'N'*-Dimethyltryptamine	66	3	14 20 14	1 1 0·8	17 20 30	— — —	1	1 — —

TABLE III

ANTAGONISTIC ACTIVITY OF SOME INDOLE DERIVATIVES ON THE GUINEA-PIG ILEUM AT 35° IN THE PRESENCE OF MORPHINE (D RECEPTORS)

Drug ratios are calculated on a molar basis, and given as means with standard errors.

Serial No.	Drug	Conc. ($\times 10^{-7}$ M)	No. of Expts.	Drug Ratio
8	5-Benzyloxygramine	3·6	2	0·92±0·28
		7·2	1	1·00
7	5-Benzyloxy-*N'N'*-dimethyltryptamine	60	3	0·33±0·1
6	*N'N'*-Dimethyltryptamine	66	3	0·055±0·002

morphine were 1.6±0.4 for 5-hydroxytryptamine and 1.4±0.4 for tryptamine (means of six experiments with standard errors), whereas at 35° they were 3.0±0.5 and 3.0±0.5 (Table I). This result was surprising. Kosterlitz and Robinson (1957, 1958) and Innes, Kosterlitz and Robinson (1957) found that morphine, atropine, and lowering the bath temperature reduced contractions of the longitudinal muscle of the guinea-pig ileum caused by 5-hydroxytryptamine. We had, therefore, expected that the responses to 5-hydroxytryptamine would be reduced even further by morphine when the bath temperature was lowered. That they were not suggested that lowering the temperature also reduced the sensitivity of the M receptors.

Investigation of the Analogues of 5-Hydroxytryptamine

Antagonistic Activity.—In the presence of morphine only three of the compounds examined (that is, on the D receptors) antagonized 5-hydroxytryptamine, and on the phenoxybenzamine-treated tissue (on the M receptors) none had any antagonistic activity (Tables II and III).

As we had hoped that tranquillizers would antagonize 5-hydroxytryptamine on the M receptors, we also tested reserpine in concentrations up to 10^{-6} M and found that it was inactive against 5-hydroxytryptamine. Higher concentrations reduced the responses to 5-hydroxytryptamine and also those to histamine and nicotine.

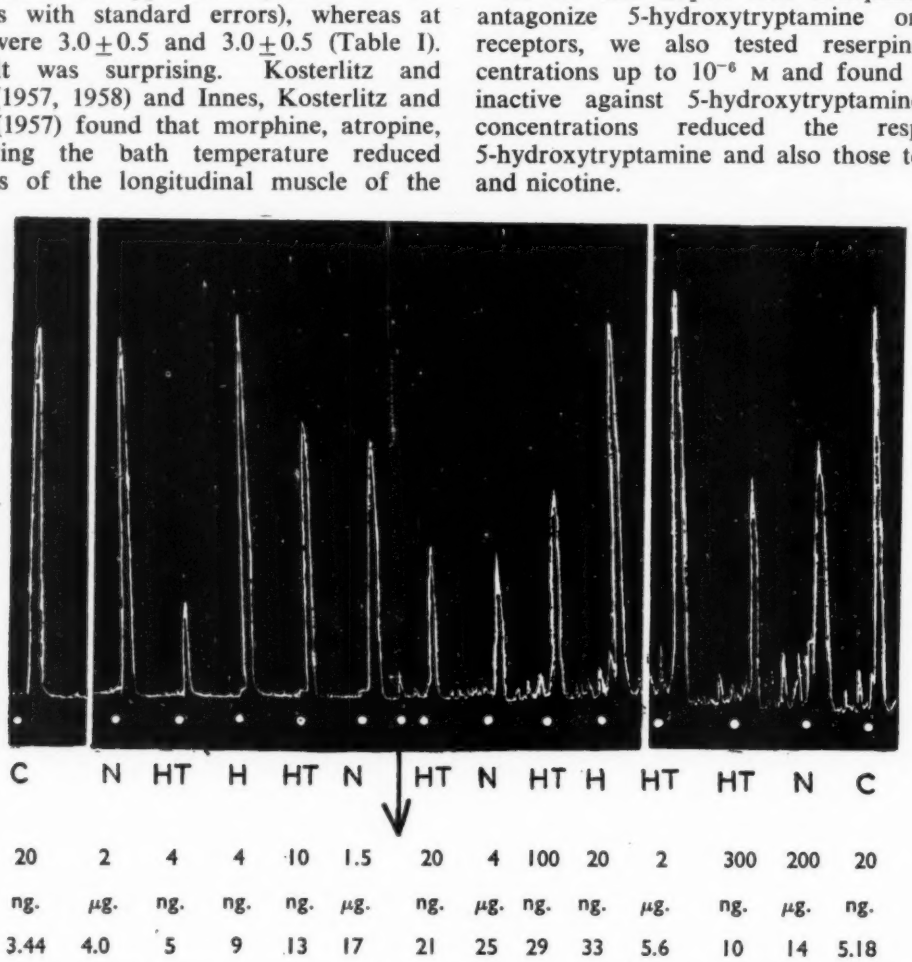


FIG. 2.—Isolated guinea-pig ileum preparation. Antagonistic effect of the analogue, 5-benzyloxy-*N'N'*-dimethyltryptamine (6.2×10^{-6} M at the arrow), on the D receptors in the presence of morphine 3.5×10^{-6} M. Responses are shown to 5-hydroxytryptamine (HT), nicotine (N), histamine (H), and carbachol (C) before and after the addition of the analogue to the bath at 4.19 p.m. At the end of 1 hr. the dose ratios were 33 for 5-hydroxytryptamine, 132 for nicotine, 20 for histamine, and 1 for carbachol. The last response to histamine is not shown in the tracing.

Two compounds tested on the D receptors had interesting side effects. First, 5-benzyloxy-*N'N'*-dimethyltryptamine (*7*) antagonized the action of nicotine (Fig. 2), which action has been termed "morphine insensitive" by Kosterlitz and Robinson (1958). Secondly, *N'N'*-dimethyltryptamine (*6*) and, to a lesser extent, 5-benzyloxy-*N'N'*-dimethyltryptamine (*7*) antagonized the action of histamine.

Stimulant Activity.—The equipotent molar ratios of the compounds tested are shown in Table IV. In the presence of morphine, contractions produced by all the stimulant compounds including 5-hydroxytryptamine and tryptamine were complete within 30 to 45 sec. Tryptamine, 5-hydroxytryptamine, and 5-hydroxy-α-methyltryptamine (*2*) all appeared to be washed out easily, but after the other compounds a slightly longer time (usually 2 min.) was required before the tissue relaxed. In concentrations used for determining the dose/response curve, the drugs produced only a single contraction of the muscle. A high concentration of *N'N'*-dimethyltryptamine (*6*) caused slight depression (lasting about 5 min.) of the control responses to 5-hydroxytryptamine, but after the other compounds the control responses were not disturbed.

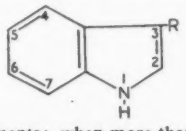
The dose/response curve of *N'N'*-dimethyltryptamine (*6*), though parallel to those of 5-hydroxytryptamine and tryptamine in low concentrations, flattened out in higher concentrations. The curves of the other compounds were parallel to those of 5-hydroxytryptamine and tryptamine over the whole range. Of the compounds tested, 5-benzyloxygramine (*8*), 5-benzyloxy-*N'N'*-dimethyltryptamine (*7*) and α-ethyltryptamine (*5*) were inactive. α-Methyltryptamine (*4*) and *N'N'*-dipropyltryptamine (*3*) were about as active as tryptamine. 5-Hydroxy-α-methyltryptamine (*2*), the most active compound, was about half as active as 5-hydroxytryptamine.

On the preparation treated with phenoxybenzamine, 5-hydroxy-α-methyltryptamine (*2*) was the only compound which was active enough for a dose/response curve to be obtained. This was parallel to that of 5-hydroxytryptamine. The other compounds tested, even in very high concentrations, produced only small effects.

Dose/response curves were obtained with a few compounds in the presence of lysergic acid

TABLE IV

STIMULANT ACTIVITY ON THE GUINEA-PIG ILEUM



Numerals in parenthesis indicate the number of experiments; when more than two were done, means and standard errors are given. Results marked with an asterisk were obtained with threshold responses; the dose/response curves could not be plotted. † Indicates that the top of the dose/response curve was not parallel to that of 5-hydroxytryptamine.

Serial No.	Ring Substituent	Side-chain (R) (Trivial Name)	Stimulant Activity (Equipotent Molar Ratios)		
			D Receptors, 35°	M Receptors, 35°	
			In the Presence of Morphine ($3{\cdot}5\times10^{-6}$M)	Phenoxybenzamine-treated Tissue	In the Presence of LSD (3×10^{-6}M)
1	5-OH	$-CH_2-CH_2-N(C_3H_7)_2$ (5-Hydroxy-*N'N'*-dipropyltryptamine)	12, 20 (2)	120±0 (2)*	12·5, 25 (2)†
2	5-OH	$-CH_2-CH(CH_3)-NH_2$ (5-Hydroxy-α-methyltryptamine)	2·3±0·03 (3)	4, 16 (2)	3·2, 8 (2)
3	—	$-CH_2-CH_2-N(C_3H_7)_2$ (*N'N'*-Dipropyltryptamine)	150±53 (4)	148, 296 (2)*	10±3·8 (4)†
4	—	$-CH_2-CH(CH_3)-NH_2$ (α-Methyltryptamine)	370±110 (3)	>200, 640 (2)*	390±125 (3)*
5	—	$-CH_2-CH(C_2H_5)-NH_2$ (α-Ethyltryptamine)	>20,000 (1)	>200 (1)	—
6	—	$-CH_2-CH_2-N(CH_3)_2$ (*N'N'*-Dimethyltryptamine)	130±40 (3)†	100 (1)*	—
7	5-OC_7H_7	$-CH_2-CH_2-N(CH_3)_2$ (5-Benzyloxy-*N'N'*-dimethyltryptamine)	5,000, >2,000 (2)*	>200 (1)	
8	5-OC_7H_7	$-CH_2-N(CH_3)_2$ (5-Benzyloxygramine)	>5,000 (1)	>200 (1)	
9	5-OH	$-CH_2-CH_2-NH_2$ (5-Hydroxytryptamine)	1	1	1
10	—	$-CH_2-CH_2-NH_2$ (Tryptamine)	162±40 (8)	360, 720 (2)*	257±30 (5)

diethylamide. The equipotent molar ratios are shown in Table IV. 5-Hydroxy-α-methyltryptamine (*2*) was again the most active compound, and its dose/response curve was parallel to those of 5-hydroxytryptamine and tryptamine. *N'N'*-Dipropyltryptamine and 5-hydroxy-*N'N'*-dipropyltryptamine (*3* and *1*) were the next most active, being stronger than tryptamine, but their dose/response curves flattened out at high concentrations. The action of these latter compounds could only be shown on the M receptors when the D receptors were blocked by lysergic acid diethylamide. Phenoxybenzamine left the tissue so insensitive (even to 5-hydroxytryptamine) that responses to the less active compounds could only be obtained with very high concentrations indeed.

One of the most striking features of Table IV is the high activity of *N'N'*-dipropyltryptamine (*3*) on the M receptors.

Effect of Higher Concentrations.—With this preparation, either in the presence of morphine or on the phenoxybenzamine-treated tissue, there was no indication with any of the compounds of the production of repeated contractions such as was seen with the rat uterus (Barlow and Khan, 1959a and b). A multiple response was sometimes seen, but the tissue rapidly relaxed in spite of the presence of the drug. The preparation was then equally insensitive to 5-hydroxytryptamine and tryptamine (even after the drug was washed out), but the control responses to nicotine, acetylcholine, and histamine were unaffected. This behaviour was also seen when lower concentrations (below those producing a maximal response) were left in the bath for a long time (more than 5 to 10 min.).

Discussion

The results of the dose/ratio experiments confirm that tryptamine and 5-hydroxytryptamine act in the same way on the "M" and "D" receptors of the guinea-pig ileum.

Estimates of the stimulant activity of analogues of 5-hydroxytryptamine on the D receptors (in the presence of morphine) resemble those on the rat uterus but not those on the rat fundus strip, on which α-methyltryptamine (*4*) and *N'N'*-dipropyltryptamine (*3*) were much more active than tryptamine (Barlow and Khan, 1959a and b). Estimates of the antagonistic activity on the D receptors also resemble those on the rat uterus.

The high activity of *N'N'*-dipropyltryptamine (*3*) on the M receptors is similar to that on the rat fundus strip, but the dose/response curve of this compound does not flatten out at high concentrations for the rat fundus strip as it does for the M receptors. None of the compounds, not even reserpine, antagonized the effects of 5-hydroxytryptamine on the M receptors, and this test does not appear to indicate any central properties the compounds may have.

The guinea-pig ileum does not seem to have any distinct advantages over the rat uterus and rat fundus strip used as a combined test for ability to imitate or antagonize the actions of 5-hydroxytryptamine. Although the guinea-pig ileum in the presence of morphine has the advantage over the rat uterus that it reveals antihistamine or antinicotine activity as well as ability to imitate or antagonize 5-hydroxytryptamine, this is offset by the disadvantage of having morphine continually present and of working with an extremely complicated tissue.

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ANTAGONISM OF THE EFFECTS OF TREMORINE BY TROPINE DERIVATIVES

BY

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Methods of testing new drugs for anti-Parkinson activity are briefly reviewed. The production in animals of Parkinson-like effects by Tremorine (1,4-dipyrrolidin-1′-ylbut-2-yne), and the inhibition of these effects in mice by a number of tropine derivatives, are described. No correlation was found between the activity against tremor and the anticholinergic, antihistaminic or local anaesthetic properties of the compounds.

During the last twelve years a number of new synthetic drugs, chiefly antispasmodics and antihistaminics, have been found useful in the treatment of Parkinson's disease, but the selection of these drugs for clinical study has been largely empirical.

Until the introduction of synthetic compounds, the natural solanaceous alkaloids were the most reliable agents in the treatment of this disease (Critchley, 1958), but the use of these drugs was complicated by undesirable side-effects such as dry mouth, blurred vision, palpitations, and nausea. An exception was tigliodine, the tiglic ester of *pseudo*tropine, which Trautner and Noack (1951) tested in a small number of patients and considered to have a similar therapeutic action to atropine but without producing the same side-effects. In 1953 Maschovsky reported the diphenylacetic ester of tropine to protect against nicotine-induced tremors and to be effective in the treatment of Parkinson's disease, and subsequently benztropine methanesulphonate was found to be one of the most effective drugs in this condition (Doshay, 1956). Both these drugs, however, caused side-effects in some patients.

These reports led us to investigate the anti-tremor properties of other tropine derivatives in the hope of finding drugs possessing antitremor activity but little antiparasympathetic action. A series of esters, mainly substituted acetyltropeïnes, was therefore prepared (Table III).

Anti-Parkinson drugs have usually been evaluated by testing them against tremors produced in experimental animals by surgical or chemical means. Jenker and Ward (1953) obtained limb movements resembling tremor by stimulating the brain-stem reticular formation of anaesthetized monkeys. Vernier and Unna (1956) produced brain-stem lesions in monkeys which caused chronic postural tremor. In both cases anti-Parkinson drugs were effective in controlling the tremor, but the limitations of these methods lie in the cost and the necessity of operating on several monkeys to produce one with tremor. Previous workers, using the rabbit, found some correlation between the ability of a compound to protect against the hyperkinetic effects of nicotine and the parkinsonian tremor, but this protective effect was seen also with a wide variety of other agents (Bovet and Longo, 1951; Cahen and Lynes, 1951; Cahen, Thomas and Tvede, 1953).

The report by Everett (1956), that Tremorine, 1,4-dipyrrolidin-1′-ylbut-2-yne, produces, particularly in monkeys, effects closely resembling those seen in the Parkinson syndrome, and that currently used anti-Parkinson compounds protect against these effects (Everett, 1956; Everett, Blockus and Shepperd, 1956; Frommel, 1958), encouraged us to use anti-Tremorine activity as a guide to the possible therapeutic value of new compounds.

METHODS

Antagonism of Tremorine.—Albino mice of the same strain weighing 18 to 22 g. were used throughout. Tremorine was injected subcutaneously in a dose of 30 mg./kg. body weight and the mice were assessed at intervals for weakness, tremor, salivation, lachrymation, rigidity, diarrhoea and excessive micturition. The assessment was on an arbitrary scale ranging from $\frac{1}{2}$ to 3. Very severe effects, such as tremors of the whole body or salivation so intense that it wet the face and neck, were scored as 3, while

slight effects, such as tremors which could be felt only in lifting the animal by the tail, or excessive saliva restricted to the mouth, were scored as ½.

Compounds under test were dissolved in water, and 0.5 ml./20 g. of body weight was injected subcutaneously 30 min. before Tremorine or given orally 1 hr. before. A group of control mice received water. Each compound was compared with caramiphen hydrochloride using ten mice at each of three dose levels. Successive doses were increased by a factor of two for compounds injected subcutaneously and three when given orally. Doses were arranged where possible to give effects varying from 0 to 100%. The score for each group of ten mice was determined for tremor and salivation and the % protection at each dose obtained from the expression 100−(score for group of treated mice/score for group of control mice)×100. The % protection obtained in this way for each dose was converted to its probit and plotted against the logarithm of the dose. Relative potency with respect to caramiphen was calculated by the method of Finney (1947).

Mydriatic Activity.—This was determined on the mouse pupil by the method of Ing, Dawes, and Wajda (1945), the diameter of the pupil being measured in groups of five mice 30 min. after intraperitoneal injection of the drug. Each compound was compared with atropine sulphate on the same day and the increase in pupil diameter plotted against the logarithm of the dose.

Anti-acetylcholine Activity.—This was measured on the isolated guinea-pig ileum. Each compound was compared with atropine sulphate (potency=1.0) on the same strip of guinea-pig ileum using a superfusion technique (Adam, Hardwick, and Spencer, 1954). The ileum was stimulated with acetylcholine (5×10^{-8}, w/v) in Tyrode solution for 15 sec. and then washed with Tyrode solution for 35 sec. Antagonists dissolved in Tyrode solution were applied to the gut manually during interruption of the flow of Tyrode solution. The % inhibitory effects of the compounds at various concentrations were plotted against the logarithm of the dose and the relative potencies of the two compounds estimated.

Acute Toxicity.—The toxicity of each compound was estimated using the same strain of mice as in the Tremorine test. Compounds were dissolved in water and injected subcutaneously into groups of five mice in a volume of 0.5 ml./20 g. of body weight. The % mortality recorded at 24 hr. for each dose was converted to the corresponding probit and plotted against the logarithm of the dose. The LD50 was estimated graphically.

Antihistamine Activity.—This was measured on the isolated guinea-pig ileum by the method used for determining anti-acetylcholine activity. The ileum was stimulated with histamine (10^{-6}, w/v) in Tyrode solution for 20 sec. and washed with Tyrode solution for 45 sec. Mepyramine maleate was used as the standard.

Local Anaesthetic Activity.—Anaesthetic potency was determined by the method of Chance and Lobstein (1944). A solution of the drug in saline was applied to the cornea of a guinea-pig and left there for 15 sec. The cornea was stimulated 45 sec. later and thereafter at 1 min. intervals for a total of 10 min., by touching it with a fine glass rod with a rounded end. Failure to blink after the stimulus counted as a positive response. Each compound was assayed against amethocaine hydrochloride using three concentrations varying by a factor of two. Both eyes of three guinea-pigs were used each day for six days and the experiment designed so that each drug solution was tested on each eye. The positive responses for each concentration of drug solution were added together at the end of the experiment, giving a maximum possible score of 60. The % effects were converted to their corresponding probits and plotted against the logarithm of the dose. Potencies were estimated graphically.

Results

Tremorine in Intact Animals.—The effects of Tremorine, whether given by the oral, intravenous, intraperitoneal or subcutaneous route, lasted for several hours and, if death had not occurred, were often seen up to 24 hr. later. After an injection of Tremorine mice developed severe tremors with fits of shaking of the whole body as well as muscular rigidity and weakness. Rigidity was very noticeable since the animals walked with a high-stepping gait, the body held well clear of the ground. In addition, signs of intense parasympathetic stimulation were evident. These were characterized by profuse salivation spreading until the jaws and neck were soaked, lachrymation, diarrhoea, micturition, and sometimes miosis. Perhaps the most striking effect was a profound fall in body temperature, sometimes by as much as 14°.

Other species behaved like mice after Tremorine. The tremors in rats after a proportionate dose of Tremorine were usually less severe than in mice. Guinea-pigs showed marked tremors and were more susceptible to the lethal effects of Tremorine, dying in 1 to 2 hr. after an equivalent dose. In rabbits the most severe effect was profuse salivation which was difficult to control even with very large doses of atropine given intravenously or subcutaneously; tremors were barely noticeable. Intraperitoneal injection of Tremorine into young chickens produced effects similar to those seen in mice.

Protection Against the Effects of Tremorine.—Results obtained with recognized anti-Parkinson compounds are shown in Table I.

TABLE I

ANTI-TREMORINE TREMOR POTENCIES, TOXICITY, AND MYDRIATIC, ANTI-ACETYLCHOLINE AND LOCAL ANAESTHETIC POTENCIES OF SOME ANTI-PARKINSON DRUGS

Anti-Tremorine tremor potencies were determined in mice injected subcutaneously with 30 mg./kg. Tremorine either 30 min. after subcutaneous injection or 60 min. after oral administration of the compounds. Each compound was assayed against caramiphen hydrochloride; the limits of the assays are given in brackets below the potencies. Trade names are given in brackets.

Compound	Potency of Tremor Inhibition (Caramiphen = 1·0)			Approx. Subcut. LD50 in Mice at 24 hr. (mg./kg.)	Mydriatic Potency in Mice 30 min. after i.p. Injection (Atropine = 1·0)	Anti-acetylcholine Potency on Guinea-pig Ileum (Atropine = 1·0)	Local Anaesthetic Potency on Guinea-pig Cornea (Amethocaine = 1·0)
	Subcutaneous Route		Oral Route				
	At 2 hr.	At 6 hr.	At 6 hr.				
Caramiphen hydrochloride (Parpanit)	1·0	1·0	1·0	400	0·01	0·9	0·1
Benzhexol hydrochloride (Artane)	8·6 (5·5–13·1)	8·0 (3·8–14·6)	2·8 (1·5–5·2)	380	0·7	0·5	<0·1
Benztropine methanesulfonate (Cogentin)	6·6 (2·8–8·4)	7·7 (4·2–9·4)	4·3 (2·3–7·7)	55	0·1	0·5	0·3
Atropine sulphate	3·2 (1·0–6·2)	2·4 (1·5–3·7)	3·0 (1·8–5·4)	610	1·0	1·0	<0·1
Ethopropazine hydrochloride (Lysivane, Parsidol)	0·8 (0·5–1·2)	1·3 (0·3–3·7)	1·2 (0·4–3·3)	670	<0·01	0·2	<0·1
Orphenadrine hydrochloride (Disipal)	0·6 (0·1–1·2)	0·4 (0·2–0·6)	0·25 (0·1–0·4)	125	0·02	0·2	0·15
Diethazine hydrochloride (Diparcol)	0·44 (0·15–1·1)	0·6 (0·5–0·9)	0·6 (0·2–2·0)	>1,000	<0·01	0·03	0·9
Diphenhydramine hydrochloride (Benadryl)	0·16 (0·03–0·3)	0·17 (0·1–0·24)	0·1 (0·07–0·17)	200	0·01	<0·01	0·1

These compounds, given orally or subcutaneously, all protected mice against Tremorine. After subcutaneous injection, maximum protection was obtained by 2 hr., and usually remained at the same level for at least a further 4 hr.; after oral administration, however, protective activity took up to 6 hr. to reach its peak. This was also found with the active tropine derivatives.

Benztropine and benzhexol were the most effective of the current anti-Parkinson compounds tested. Caramiphen, which was used as the standard throughout, had an antitremor ED50 (mean of all assays) of 7.5 mg./kg. at 2 hr. and 6.4 mg./kg. at 6 hr. after subcutaneous injection, while at 6 hr. after oral administration the ED50 was 6.8 mg./kg.

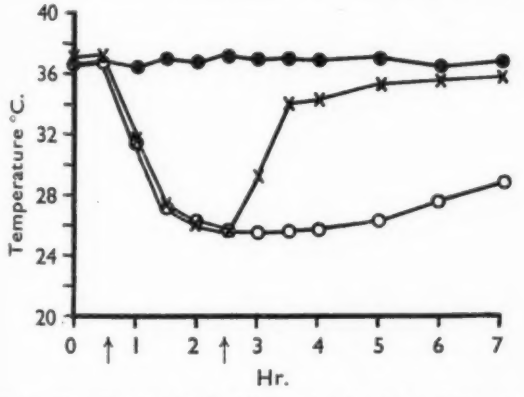

FIG. 1.—Hypothermic effect of Tremorine in mice and its reversal by caramiphen hydrochloride. Each point represents mean response of five mice. Room temperature was 22°. Arrows indicate times of injection. ●—●, saline 25 ml./kg. followed by caramiphen 20 mg./kg. X--X, Tremorine 30 mg./kg. followed by caramiphen 20 mg./kg. O—O, Tremorine 30 mg./kg. followed by saline 25 ml./kg.

On the whole the active compounds appeared to control tremor, rigidity, weakness, and parasympathomimetic effects simultaneously and to the same degree. Hypothermia was most difficult to control and, even with large doses of protective drugs, some fall in temperature could often be recorded after an injection of Tremorine. The action of caramiphen in reversing hypothermia due to Tremorine is illustrated in Fig. 1. Caramiphen alone had no effect on the temperature of normal mice.

Several compounds having different types of pharmacological properties (Table II) were found

TABLE II

COMPOUNDS WITHOUT ANTI-TREMORINE TREMOR ACTIVITY

Compounds were injected subcutaneously into mice 30 min. before Tremorine, 30 mg./kg. subcutaneously, and effects noted for up to 6 hr.

Compound	Greatest Dose with No Activity (mg./kg.)
Tropine hydrochloride	50
Homatropine ,, ...	50
Tigliodine hydrobromide	50
Tiglyltropeine ,, ...	50
Tubocurarine chloride	0·05
Mephenesin carbamate	50
Hexamethonium bromide	50
Morphine hydrochloride	50
Methylpentynol	20
Procaine hydrochloride	100
Lignocaine ,, ...	50

TABLE III

ANTI-TREMORINE TREMOR POTENCIES, TOXICITY, AND MYDRIATIC, ANTI-ACETYLCHOLINE AND LOCAL ANAESTHETIC POTENCIES OF SOME TROPINE DERIVATIVES

Anti-Tremorine tremor potencies were determined in mice injected subcutaneously with 30 mg./kg. Tremorine either 30 min. after subcutaneous injection or 60 min. after oral administration of the compounds. Each compound was assayed against caramiphen hydrochloride; the limits of the assays are given in brackets below the potencies. Asterisks indicate compounds which damaged the guinea-pig cornea during local anaesthetic test. Tr indicates tropane nucleus.

DF No.	Chemical Name	$R_1R_2R_3C{\cdot}CO{\cdot}O{\cdot}Tr$, HCl			Potency of Tremor Inhibition (Caramiphen = 1·0)			Approx. Subcut. LD50 in Mice at 24 Hr. (mg./kg.)	Mydriatic Potency in Mice 30 min. after i.p. Injection (Atropine = 1·0)	Anti-acetylcholine Potency on Guinea-pig Ileum (Atropine = 1·0)	Local Anaesthetic Potency on Guinea-pig Cornea (Amethocaine = 1·0)
					Subcutaneous Route		Oral Route				
		R_1	R_2	R_3	At 2 Hr.	At 6 Hr.	At 6 Hr.				
258	Acetyltropeine	H–	H–	H–	<0·05	<0·05	<0·05	500	<0·01	<0·01	<0·1
256	Phenylacetyltropeine ..	H–	H–	C_6H_5–	<0·1	<0·1	—	255	0·01	<0·01	0·01
255	Diphenylacetyltropeine ..	H–	C_6H_5–	C_6H_5–	0·9 (0·3–1·8)	2·5 (1·9–3·4)	0·9 (0·4–1·1)	155	0·02	0·9	0·2
333	Diphenylacetyl*pseudo*tropeine	H–	C_6H_5–	C_6H_5–	0·3 (0·2–0·4)	0·2 (0·1–0·4)	—	200	0·01	0·1	0·2
286	Hexylphenylacetyltropeine ..	H–	C_6H_5–	CH_3–$[CH_2]_5$–	0·3 (0·2–0·5)	<0·1	<0·1	250	<0·01	<0·01	<0·1*
20	Cyclohexylphenylacetyltropeine	H–	C_6H_5–	Cyclohexyl	2·8 (0·9–6·1)	4·5 (2·0–9·2)	3·1 (1·7–6·0)	140	0·06	0·2	0·6
218	Naphth-1-ylphenylacetyltropeine	H–	C_6H_5–	Naphth-1-yl	<0·1	<0·1	—	170	<0·01	<0·01	0·2
303	*p*-Chlorodiphenylacetyltropeine	H–	C_6H_5–	*p*–Cl–C_6H_4–	1·0 (0·5–1·7)	0·9 (0·2–2·2)	0·7 (0·4–1·2)	310	<0·01	0·2	≏0·5*
458	*o*-Methoxydiphenylacetyltropeine	H–	C_6H_5–	*o*–CH_3O–C_6H_4–	0·3 (0·07–0·5)	0·5 (0·3–0·8)	—	185	<0·01	0·02	0·1
346	Phenyl-*p*-tolylacetyltropeine	H–	C_6H_5–	*p*–CH_3–C_6H_4–	<0·1	<0·1	—	190	<0·01	0·3	0·2
280	Cyclohexylnaphth-1-ylacetyltropeine	H–	Cyclohexyl	Naphth-1-yl	<0·2	<0·2	—	>500	<0·01	<0·01	<0·1*
287	Di(*p*-chlorophenyl)acetyltropeine	H–	*p*–Cl–C_6H_4–	*p*–Cl–C_6H_4–	<0·2	<0·2	—	>500	<0·01	<0·01	<0·1*
309	*αα*-Diphenylpropionyltropeine	CH_3–	C_6H_5–	C_6H_5–	1·4 (0·8–2·7)	1·9 (0·9–3·7)	0·7 (0·4–1·1)	220	0·08	0·5	0·5
459	*αα*-Diphenylbutyryltropeine..	C_2H_5–	C_6H_5–	C_6H_5–	<0·1	<0·1	—	>150	0·01	0·04	0·4
382	*αα*-Diphenylvaleryltropeine ..	CH_3–$[CH_2]_2$–	C_6H_5–	C_6H_5–	0·5 (0·2–1·0)	0·3 (0·2–0·5)	—	250	<0·01	<0·01	≏0·5*
462	*αα*-Diphenylisovaleryltropeine	$(CH_3)_2CH$–	C_6H_5–	C_6H_5–	<0·1	<0·1	—	215	<0·01	<0·01	—
254	Diphenylglycolloyltropeine ..	HO–	C_6H_5–	C_6H_5–	5·0 (2·9–9·1)	6·4 (4·2–11·0)	1·3 (0·8–2·0)	150	1·0	1·0	0·2
549	Methoxydiphenylacetyltropeine	CH_3O–	C_6H_5–	C_6H_5–	1·1 (0·6–1·7)	2·0 (1·5–2·6)	3·4 (1·0–10·5)	200	0·02	0·2	≏0·2
452	Ethoxydiphenylacetyltropeine	C_2H_5O–	C_6H_5–	C_6H_5–	3·7 (1·1–6·6)	3·1 (1·8–4·7)	5·8 (2·4–7·6)	170	0·07	0·04	0·3
556	Diphenylpropoxyacetyl-	CH_3–$[CH_2]_2$–O–	C_6H_5–	C_6H_5–	3·4 (2·0–8·0)	3·7 (2·1–10·5)	2·3 (1·1–4·3)	170	0·04	<0·01	0·5

452	Ethoxydiphenylacetyltropeïne	C_2H_5O-	C_6H_5-	C_6H_5-											
556	Diphenylpropoxyacetyltropeïne	CH_3-$[CH_2]_2$-O-	C_6H_5-	C_6H_5-		3·4 (2·0–8·0)	3·7 (1·1–6·6)	3·7 (2·1–10·5)	3·1 (1·8–4·7)	2·3 (1·1–4·3)	5·8 (2·4–7·6)	170	0·04	< 0·01	0·5
550	Phenoxydiphenylacetyltropeïne	C_6H_5-O-	C_6H_5-	C_6H_5-		1·1 (0·3–2·0)		2·7 (1·7–4·1)		1·2 (0·2–3·5)		300	< 0·01	< 0·01	0·3
555	Benzyloxydiphenylacetyltropeïne	C_6H_5-CH_2-O-	C_6H_5-	C_6H_5-		2·8 (1·0–6·6)		4·2 (2·0–10·9)		7·8 (5·8–9·0)		> 500	< 0·01	< 0·01	—*
		n													
272	1-Phenylcyclopropane-1-carbonyltropeïne		2			0·2 (0·1–0·4)		0·3 (0·2–0·5)		—		135	< 0·01	0·5	0·06
263	1-Phenylcyclopentane-1-carbonyltropeïne		4			4·5 (2·0–9·2)		5·0 (3·0–8·4)		5·0 (3·6–7·0)		230	0·08	0·5	≦0·5*
288	1-Phenylcyclohexane-1-carbonyltropeïne		5			4·1 (1·9–7·0)		4·4 (1·8–9·9)		17·0 (12·2–25·6)		300	0·05	0·2	0·3
721	3-o-Methyldiphenylmethoxytropane					5·1 (3·2–8·1)		4·4 (2·7–6·5)		1·7 (0·5–5·5)		43	0·03	0·3	0·6

to be ineffective in antagonizing the action of Tremorine, thus confirming the results of Everett (1956). Hexobarbitone and ether in doses causing anaesthesia did control tremors and rigidity, though some twitching, mainly of the tail, was seen even when reflex movements to a painful stimulus were absent.

Structure / Activity Relationships. — The results of modification of the structure of acetyltropeïne can be seen in Table III. Acetyltropeïne (DF258) did not control tremors. Substitution of one hydrogen atom of the acyl radical by a phenyl group still gave an inactive compound (DF256), but substitution of two hydrogen atoms by two phenyl groups (DF255) gave moderate activity against tremors. There was a fall in activity with the isomeric *pseudo*tropine ester (DF333, diphenylacetyl*pseudo*tropeïne). If one phenyl group was present then replacement of the second hydrogen atom by an aliphatic radical (DF286, hexylphenylacetyltropeïne) gave slight activity, but the presence of a suitably located hydroxyl group as in atropine (Table I) raised it still further. Replacement of one phenyl group in diphenylacetyltropeïne by a cyclohexyl group (DF20) substantially increased activity although a similar replacement by an α-naphthyl group (DF218), or replacement of both phenyl groups by a cyclohexyl group and an α-naphthyl group (DF280, cyclohexylnaphth-1-ylacetyltropeïne), was disadvantageous. In general monosubstitution in one or both phenyl groups (DF303, *p*-chlorodiphenylacetyltropeïne ; DF458, *o*-methoxydiphenylacetyltropeïne ; DF346, phenyl-*p*-tolylacetyltropeïne ; and DF287, di(*p*-chlorophenyl)-acetyltropeïne) also considerably depressed activity.

There was no advantage in replacing the third hydrogen atom in the acyl group of diphenylacetyltropeïne by a straight-chain or branched-chain aliphatic residue (DF309, αα-diphenylpropionyltropeïne ; DF459, αα-diphenylbutyryltropeïne ; DF382, αα-diphenylvaleryltropeïne ; and DF462, diphenylisovaleryltropeïne), but introduction of a hydroxyl group at this point enhanced potency considerably (DF254, diphenylglycolloyltropeïne). The effect of substitution in this tertiary hydroxyl group by an alkyl, aryl or aralkyl group was to reduce

TABLE IV

ANTI-TREMORINE TREMOR POTENCIES, TOXICITY, AND MYDRIATIC, ANTI-ACETYLCHOLINE AND LOCAL ANAESTHETIC POTENCIES OF A SERIES OF KNOWN ALIPHATIC AMINOALCOHOLS

Anti-Tremorine tremor potencies were determined in mice injected subcutaneously with 30 mg./kg. Tremorine either 30 min. after subcutaneous injection or 60 min. after oral administration of the compounds. Each compound was assayed against caramiphen hydrochloride; the limits of the assays are given in brackets below the potencies.

Compound	$R_1R_2R_3C{\cdot}CO{\cdot}O{\cdot}CH_2{\cdot}CH_2{\cdot}N[C_2H_5]_2$			Potency of Tremor Inhibition (Caramiphen = 1·0)			Approx. Subcut. LD50 in Mice at 24 hr. (mg./kg.)	Mydriatic Potency in Mice 30 min. after i.p. Injection (Atropine = 1·0)	Anti-acetylcholine Potency on Guinea-pig Ileum (Atropine = 1·0)	Local Anaesthetic Potency on Guinea-pig Cornea (Amethocaine = 1·0)
				Subcutaneous Route		Oral Route				
	R_1	R_2	R_3	At 2 Hr.	At 6 Hr.	At 6 Hr.				
Adiphenine	H–	C_6H_5–	C_6H_5–	< 0·05	< 0·05	< 0·05	400	0·01	0·01	< 0·1
Trasentin 6H	H–	C_6H_5–	Cyclohexyl	0·7 (0·4–1·1)	1·1 (0·5–2·9)	0·6 (0·3–1·0)	500	0·01	0·01	0·1
Benactyzine	HO–	C_6H_5–	C_6H_5–	1·2 (0·7–1·6)	0·9 (0·3–1·4)	0·9 (0·3–1·1)	250	0·08	1·0	< 0·1

greatly the peripheral anti-acetylcholine activity whilst maintaining to a large extent the antitremor activity (DF549, methoxydiphenylacetyltropeïne; DF452, ethoxydiphenylacetyltropeïne; DF556, diphenylpropoxyacetyltropeïne; DF550, phenoxydiphenylacetyltropeïne; and DF555, benzyloxydiphenylacetyltropeïne).

A few tropine esters of 1-phenylcycloalkane-1-carboxylic acids were also examined. The size of the cycloalkyl ring was of importance in determining antitremor activity since the cyclopentane (DF263) and cyclohexane (DF288) derivatives were considerably more active than the cyclopropane analogue (DF272).

It was of interest to determine whether replacement of the diethylaminoethyl group by a tropyl radical in compounds of the type used in Parkinson's disease would have advantages. Results obtained with adiphenine (Trasentin), Trasentin 6H and benactyzine are shown in Table IV. Comparison of their properties with those of the corresponding tropine analogues DF255, DF20, and DF254 shows that the use of tropine increases potency against tremors but that the toxicity is also increased, though not to the same extent. Similar comparison between compounds derived from tropine and those derived from aliphatic aminoalcohols may be made between diphenhydramine and benztropine (Table I), caramiphen (Table I) and DF263 (Table III), and orphenadrine (Table I) and DF721 (3-*o*-methyldiphenylmethoxytropane) (Table III). In each of these instances the tropane analogue is both more potent and more toxic.

Effects of Quaternization.—A number of the compounds containing a tertiary nitrogen atom were converted to quaternary salts, such as the methiodide or methobromide, and tested against Tremorine. With these compounds inhibition of tremor was slow to appear and even 6 hr. after subcutaneous injection was usually not as great as that seen with a corresponding dose of the tertiary compound (Table V). However, these compounds readily controlled the parasympathomimetic effects and a dose could be found which completely inhibited these effects whilst having no action on the tremors.

TABLE V

COMPARISON AS TREMORINE ANTAGONISTS OF PAIRS OF COMPOUNDS CONTAINING TERTIARY OR QUATERNARY NITROGEN ATOMS

Compounds injected subcutaneously into groups of five or ten mice 30 min. before Tremorine, 30 mg./kg. subcutaneously. Pairs of compounds were tested on different days. Anti-acetylcholine potency was measured on guinea-pig ileum relative to atropine sulphate (= 1·0).

Compound	DF No.	Salt	Approx. ED50 for Tremorine Tremor Inhibition (mg./kg.)		Anti-acetylcholine Potency
			At 2 hr.	At 6 hr.	
Atropine	—	Sulphate	2·0	1·5	1·0
	80	Methiodide	10·0	1·5	1·3
Cyclohexylphenylacetyltropeïne	20	HCl	2·0	1·0	0·2
	261	Methobromide	20·0	7·0	0·1
p-Chlorodiphenylacetyltropeïne	303	HCl	8·0	4·5	0·2
	302	Methobromide	30·0	12·0	0·4
αα-Diphenylpropionyltropeïne	309	HCl	9·0	7·0	0·5
	310	Methobromide	50·0	50·0	0·03
Diphenylglycolloyltropeïne	254	HCl	2·0	2·0	1·0
	262	Methobromide	4·0	1·0	1·5
Ethoxydiphenylacetyltropeïne	452	HCl	3·0	2·0	0·04
	451	Methobromide	45·0	10·0	0·07
1-Phenylcyclopentane-1-carbonyltropeïne	263	HCl	3·0	1·5	0·5
	264	Methobromide	12·0	12·0	1·8
Methanthelinium ..	—	Bromide	50·0	26·0	4·0

Anti-acetylcholine Activity.—There was no correlation between the anti-acetylcholine and the antitremor activities of the compounds (Table III) whether measured against acetylcholine contractions of the guinea-pig ileum or by mydriasis of the mouse pupil.

Acute Toxicity.—Subcutaneous administration to mice of large doses of derivatives of acetyltropeïne produced hypotonia as well as tremors and convulsions, but these latter effects were reduced or abolished when two of the hydrogen atoms of the acyl group were replaced by two phenyl groups (DF255), a phenyl and a *p*-chlorphenyl group (DF303), two *p*-chlorophenyl groups (DF287), a phenyl and a hexyl group (DF286), or a phenyl and a cyclohexyl group (DF20), and when three of the hydrogen atoms are replaced by a methyl and two phenyl groups (DF309) or a hydroxyl and two phenyl groups (DF254).

Toxic doses of most of the compounds caused the appearance of red urine which varied from pale pink to bright red; this was also found with some of the known anti-Parkinson drugs, for example, orphenadrine and benztropine. This colour was shown both by chemical and spectrophotometric tests to be due to haemoglobin, but few blood cells were seen on microscopic examination. The plasma of heparinized blood removed from animals showing haemoglobinuria was distinctly red while that from control animals was colourless.

Local Anaesthetic Activity.—A considerable number of tropine derivatives were found to possess surface anaesthetic activity. Some of the compounds damaged the cornea at concentrations below or about anaesthetic level (Table III). There was no correlation between antitremor and local anaesthetic activity.

Antihistamine Activity.—Very little antihistamine activity was seen with any of the tropine derivatives with the exception of diphenylacetyltropeïne, which was about one-tenth as active as mepyramine maleate.

Discussion

All methods of testing drugs in animals for possible anti-Parkinson activity have limitations, and the results often show discrepancies from those found clinically. Bovet and Longo (1951) found some correlation between antagonism of nicotine-induced tremors and clinical effectiveness of anti-Parkinson drugs, but atropine, which is useful clinically, was found to be ineffective against nicotine-induced tremors. Cahen *et al.* (1953), on the other hand, found that some anti-adrenaline agents inhibited nicotine-induced tremors in the rabbit. The experiments of Jenker and Ward (1953) on anaesthetized monkeys also showed certain discrepancies from clinical experience; scopolamine, diethazine, amyl nitrite and some quaternary nitrogen derivatives of phenothiazine were effective in monkeys although only the first two are useful clinically. Vernier and Unna (1956) found that the technique of assaying drugs in monkeys with chronic tremor induced by brain-stem lesions gave extremely good qualitative agreement with clinical testing of anti-Parkinson compounds as far as tremor was concerned, but this method did not measure the effect on rigidity. Another disadvantage was that active compounds often caused sleep or depression in these monkeys.

The use of Tremorine suggests a much easier method of screening compounds for anti-Parkinson activity. The present work shows that the recognized anti-Parkinson drugs are all highly effective against Tremorine-induced tremors. This confirms results by Everett (1956), Everett *et al.* (1956) and Frommel (1958). The chief advantage of the Tremorine technique is that it consistently produces effects in small animals similar to those seen clinically in Parkinson's disease. Doubt has been cast on the validity of the Tremorine test by Trautner and Gershon (1959), based mainly on the fact that tigliodine is ineffective in this test. Trautner and Gershon (1959) also confirm the report by Everett (1956) that quaternary aminoalkyl esters such as methanthelinium antagonize only the parasympathomimetic effects of Tremorine without affecting the tremor, and they therefore agree with Everett (1956) that the central and parasympathomimetic effects of Tremorine can be separated. The results in Table V show that quaternary compounds can in fact antagonize tremor though doses can be found which show preferential antagonism of the parasympathetic effects. These compounds take longer to act than the corresponding tertiary compounds and greater doses are required for the same protective effect. These results suggest that the tremors and parasympathomimetic effects of Tremorine may both be centrally mediated, but that the latter actions can be antagonized peripherally as well as centrally.

Although the symptoms of Parkinsonism are believed to be of purely central origin most of the drugs commonly used in this disease have considerable peripheral anti-acetylcholine activity (Table I). Thus the difficulty in employing such drugs clinically is reflected in the frequent criticism

TABLE VI

RATIO (R) OF ANTI-TREMORINE ACTIVITY TO PERIPHERAL ANTI-ACETYLCHOLINE ACTIVITY

Anti-Tremorine tremor activity was measured in mice 6 hr. after oral administration of compounds. 30 mg./kg. Tremorine was injected subcutaneously 1 hr. after compounds. Caramiphen hydrochloride (=1·0) was used as standard. Anti-acetylcholine activity was measured on guinea-pig ileum using atropine sulphate (=1·0) as standard. See text for ratio R.

DF No.	Compound	Ratio R
—	Benactyzine hydrochloride	0·9
255	Diphenylacetyltropeïne hydrochloride	1·0
—	Caramiphen hydrochloride	1·1
—	Orphenadrine ,,	1·3
254	Diphenylglycolloyltropeïne hydrochloride	1·3
309	αα-Diphenylpropionyltropeïne ,,	1·4
—	Atropine sulphate	3·0
303	p-Chlorodiphenylacetyltropeïne hydrochloride	3·5
—	Benzhexol hydrochloride	5·6
721	3-o-Methyldiphenylmethoxytropane hydrochloride	5·7
—	Ethopropazine hydrochloride	6·0
—	Benztropine methanesulphonate	8·6
—	Diphenylhydramine hydrochloride	>10·0
263	1-Phenylcyclopentane-1-carbonyltropeïne hydrochloride	10·0
20	Cyclohexylphenylacetyltropeïne hydrochloride	15·5
549	Methoxydiphenylacetyltropeïne ,,	17·0
—	Diethazine hydrochloride	21·0
—	Trasentin 6H	60·0
288	1-Phenylcyclohexane-1-carbonyltropeïne hydrochloride	85·0
550	Phenoxydiphenylacetyltropeïne hydrochloride	>120·0
452	Ethoxydiphenylacetyltropeïne ,,	145·0
556	Diphenylpropoxyacetyltropeïne ,,	>230·0
555	Benzyloxydiphenylacetyltropeïne ,,	>780·0

that salivation is controlled more readily than tremor or rigidity, or, alternatively, that in order to exercise reasonable control over the latter symptoms, undesirable side-effects become prominent. One possibility of improving these compounds was to reduce selectively this peripheral anti-acetylcholine action. With several compounds it was possible to do this and at the same time increase the antitremor potency by replacing the aliphatic aminoalcohol radical by tropine. The results of modification to the acetyltropeïne structure indicate that anti-Tremorine activity may be obtained with very little associated peripheral anti-acetylcholine activity. It is evident from consideration of Tables I, III, and IV that peripheral anti-acetylcholine activity is not a reliable guide to central activity as measured by inhibition of tremors produced by Tremorine. If attention is confined to the most potent drugs, and if the arbitrary ratio R (tremor inhibition 6 hr. after an oral dose/the anti-acetylcholine activity on the guinea-pig ileum, both expressed as ratios of controls using standard drugs) is calculated, some interesting variations are seen (Table VI). At the low end of the scale, ratios between 0.9 and 3 are given by benactyzine, diphenylacetyltropeïne (DF255), caramiphen, orphenadrine, diphenylglycolloyltropeïne (DF254), αα-diphenylpropionyltropeïne (DF309) and atropine, and at the high end of the scale, ratios of over 100 are given by the ethers of benziloyltropeïne with the exception of the methyl ether. The value of 85 for the tropine ester of 1-phenylcyclohexane-1-carboxylic acid (DF288) is not far behind. From this point of view it seems that some of the compounds having a low peripheral effect (high value of R) are worthy of clinical trial in patients with Parkinson's disease.

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COMPARISON OF EFFECTS OF VALYL³-OXYTOCIN AND SYNTOCINON ON THE CARDIO-VASCULAR SYSTEM OF MAN

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Valyl³-oxytocin has similar circulatory effects in man to those of Syntocinon (synthetic oxytocin). On intravenous administration, it caused a rise in limb blood flow and in pulse rate and a fall in blood pressure. It had a direct vasodilator effect on limb blood vessels. Valyl³-oxytocin was 1.5 to 2 times more potent than Syntocinon in terms of its circulatory effect in man. The circulatory effects of valyl³-oxytocin were less readily antagonized by vasopressin than are those of Syntocinon.

It has been shown recently by Kitchin, Lloyd, and Pickford (1959) that both Pitocin and synthetic oxytocin (Syntocinon), administered intravenously, cause an increase in limb blood flow, accompanied by a fall in blood pressure and a rise in pulse rate and cardiac output. The vasodilatation in the limbs appears to be due to a direct action of the hormone on blood vessels.

Valyl³-oxytocin is one of a series of structural analogues of oxytocin recently described by Boissonnas, Guttmann, Jacquemond, Waller, Konzett, and Berde (1956). Its chemical composition is

Cys-Tyr-Val-Glu(NH_2)-Asp(NH_2)-Cys-Pro-Leu-Gly(NH_2)

It differs from synthetic oxytocin only in the substitution of a valyl group for the isoleucyl group. When given in terms of doses assayed by the pharmacopoeial methods for oxytocin, valyl³-oxytocin was found to be several times more active than Syntocinon in its effect on the human uterus (Smyth, 1958). The object of the present experiments was to compare its cardio-vascular actions in man with those of Syntocinon and to determine whether the two substances were qualitatively similar in their effects and, if so, their relative potency.

METHODS

Observations were made on 10 healthy subjects ranging from 20 to 61 years of age. They were lightly clad and lay on a couch in a room at 18 to 20°. During the experiments, a slow intravenous infusion of saline was maintained (1.5 ml./min.) into the infusion system from a constant-speed motor-driven syringe, and the test substances were administered through the same system either as a single injection or as a constant infusion. The time of administration of the substances was unknown to the subject.

Both the preparations used, Syntocinon and valyl³-oxytocin, were supplied by Sandoz Ltd.

The standardization of valyl³-oxytocin was carried out by the method of the British Pharmacopoeia (1958), that is the effect on the blood pressure of the chicken and on the isolated rat uterus, in comparison with International Standard Pituitary (Posterior Lobe) Powder. In the present series of experiments when comparing the effects of oxytocin and of the analogue, the injections were given in pairs, using the same dose of each substance. The order was varied in different subjects in order to try to compensate for the tendency to a diminishing response with repeated administration. Intra-arterial injections were also given in nine subjects through the indwelling intra-brachial needle used for blood pressure measurements.

Hand and forearm blood flow was recorded by venous occlusion plethysmography, the water in the plethysmographs being kept at 32 to 34°. Flows were recorded at half-minute intervals, and more frequently following injections. *Blood pressure* was recorded in nine cases by indwelling intra-brachial arterial needle connected to a Minirack electromanometer (Southern Instruments) and photographically-recording Elmquist galvanometer. *Pulse rate* changes were read from plethysmograph or blood pressure tracings.

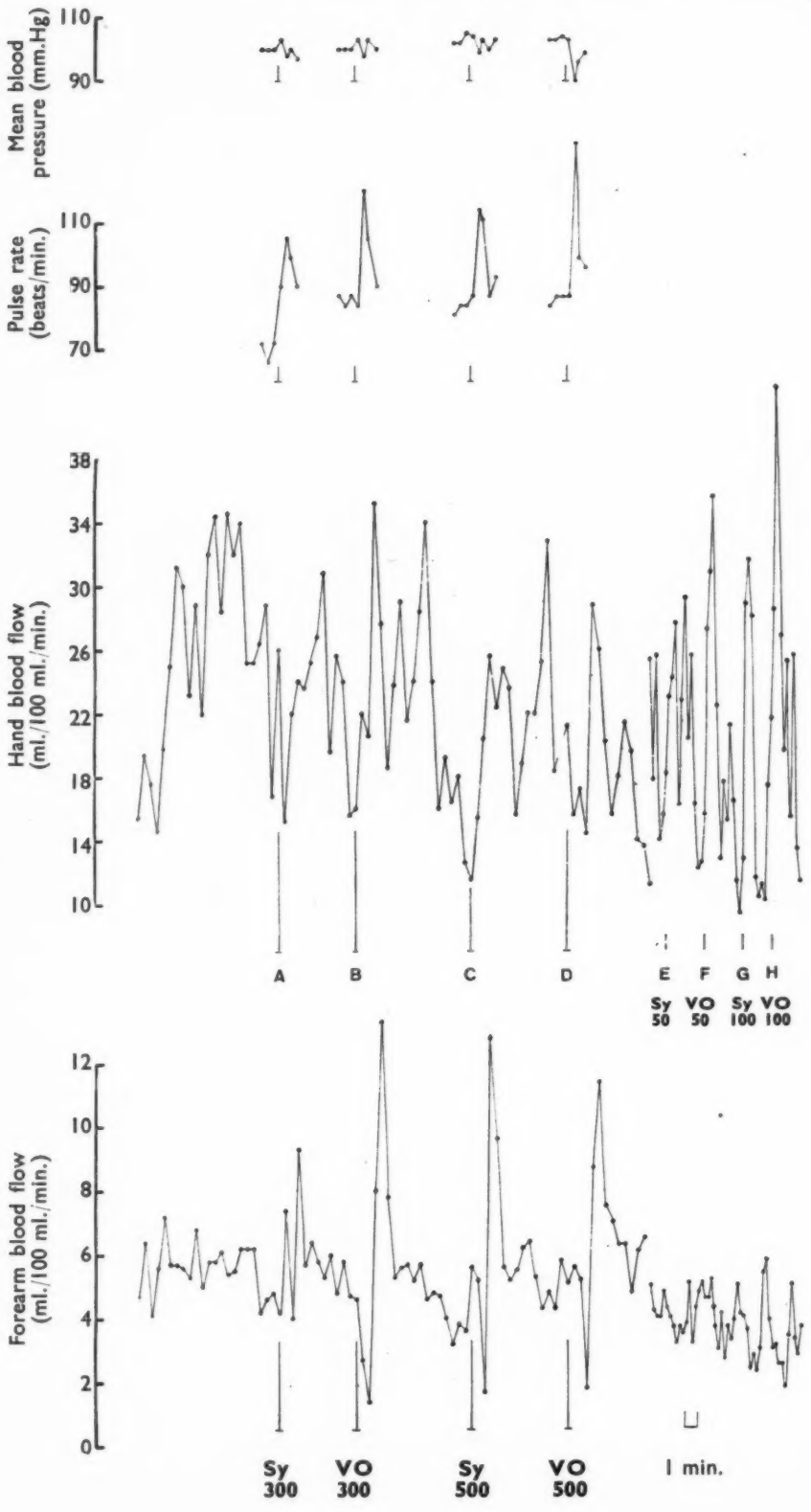

FIG. 1.—The effect in one subject of single injections of valyl³-oxytocin (**VO**) and Syntocinon (**Sy**). The records from above downwards are of mean blood pressure in mm. Hg; pulse rate in beats/min.; blood flow through hand and forearm in ml./100 ml. of tissue volume/min. Intravenous injections given at **A, B, C** and **D** and intra-brachial artery injections at **E, F, G** and **H**. The numerals under **VO** and **Sy** indicate the dose in mU.

RESULTS

Fig. 1 shows the response of one subject to single intravenous injections of valyl³-oxytocin and Syntocinon. It can be seen that the effects of the two polypeptides were qualitatively similar, and this held true in all subjects; both substances caused a rise in blood flow in the hand and forearm, a rise in pulse rate, and a small drop in blood pressure. The duration of the effects of the two substances was short and similar. The threshold doses were about 200 mU. for a single intravenous injection. On intra-arterial injection, both drugs caused a vasodilatation in the hand and forearm, sometimes with as little as 20 mU. (see Figs. 1, 2, and 3). With continuous intravenous infusions of 1 U./min., the pattern of response was again similar in the two drugs (Fig. 2), the vasodilator and hypotensive effect of valyl³-oxytocin usually being greater than that of Syntocinon.

In a previous paper (Kitchin *et al.*, 1959) it was shown that the dilator effect of infusions of

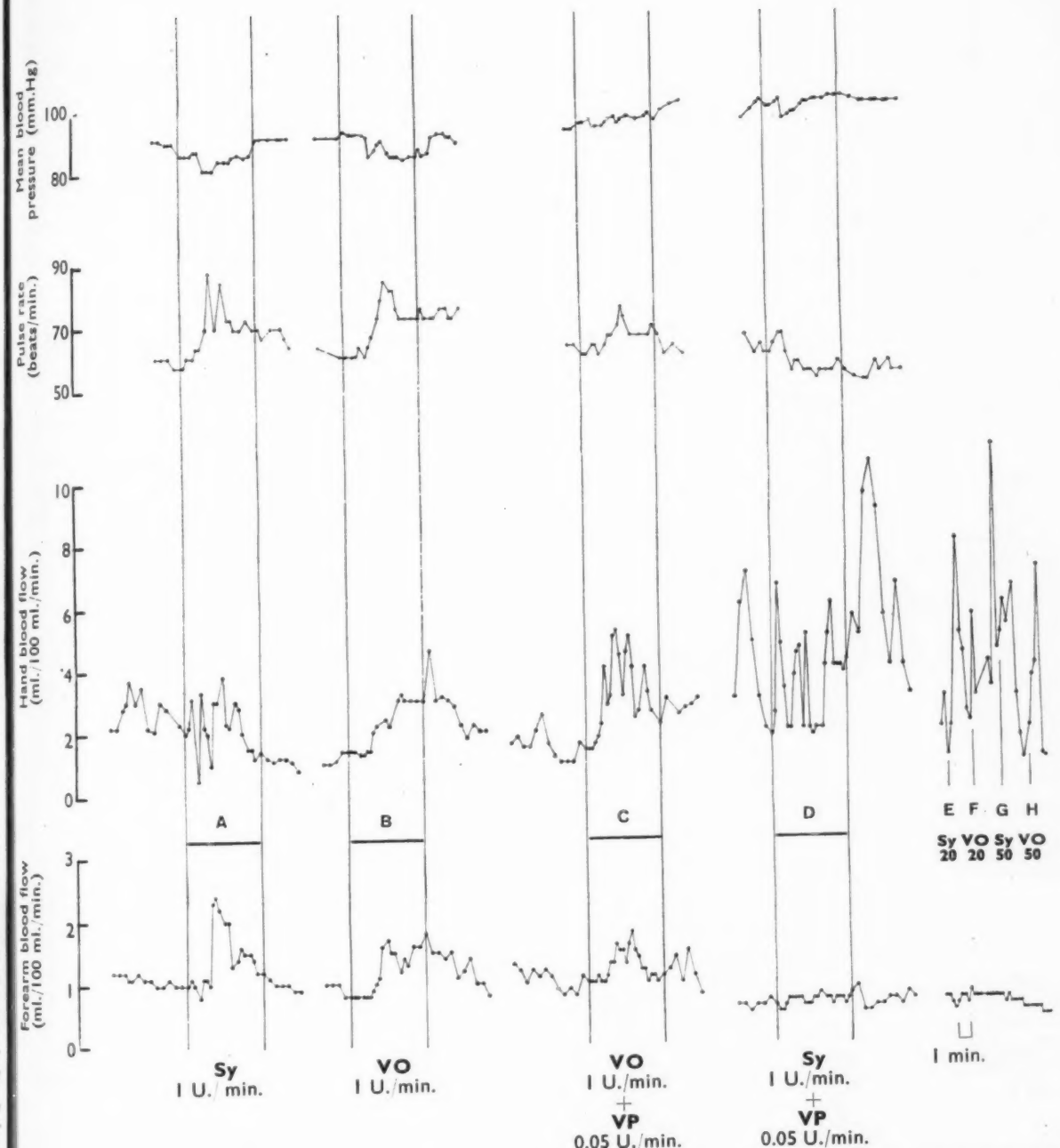

FIG. 2.—The effect in one subject of infusions of valyl³-oxytocin (**VO**) and Syntocinon (**Sy**). The records from above downwards are of mean blood pressure in mm. Hg; pulse rate in beats/min.; blood flow through hand and forearm in ml./100 ml. tissue volume/min. Intra-brachial artery single injections given at **E**, **F**, **G** and **H**. The infusions given between vertical lines marked **A**, **B**, **C** and **D**. The numerals under these capitals indicate the dose in mU. During periods **C** and **D**, vasopressin (**VP**) was added to the infusion fluid.

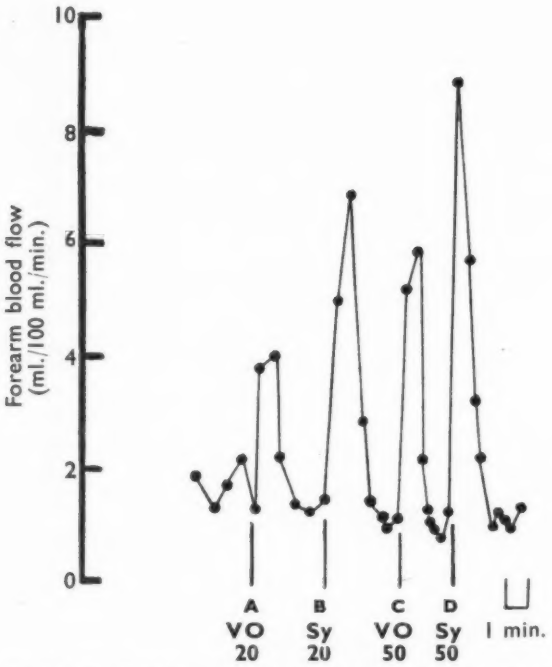

FIG. 3.—The effect in one subject of intra-arterial injections of valyl[3]-oxytocin (VO) and Syntocinon (Sy). The record is of the blood flow through the forearm in ml./100 ml. tissue volume/min. Intra-brachial artery injections given at A, B, C and D. The numerals under these capitals indicate the dose in mU.

Syntocinon was abolished by adding to the infusion fluid 1/20 the number of units of vasopressin. These experiments were repeated and a comparison made of the effect of infusions of Syntocinon and valyl[3]-oxytocin, to both of which 50 mU./min. of vasopressin had been added. The dilator effect of Syntocinon was largely abolished, while that of valyl[3]-oxytocin was reduced to a much lesser extent (Fig. 2). This difference was noted in both the subjects in whom this test was performed, and confirms the previous impression that valyl[3]-oxytocin is a stronger vasodilator and hypotensive agent than Syntocinon.

Quantitatively, the responses to valyl[3]-oxytocin were in most cases greater than those of Syntocinon given in the same doses. Table I shows as percentages the average amount by which the response to valyl[3]-oxytocin exceeded that of Syntocinon when intravenous injection and infusion as well as intra-arterial injection was used. It will be seen that, when given by intravenous injection and infusion, valyl[3]-oxytocin had a considerably greater effect on pulse rate, hand and forearm blood flow than had Syntocinon; its blood pressure lowering effect was also somewhat greater. Given by the intra-arterial route, valyl[3]-oxytocin also increased hand blood flow more than Syntocinon, but, on the forearm blood flow, Syntocinon appeared to be the more potent vasodilator (Fig. 3 and Table I). Only two pairs of observations were made, but they may be significant in view of the consistent results obtained in the hand.

TABLE I

PERCENTAGE BY WHICH THE RESPONSE TO VALYL[3]-OXYTOCIN DIFFERED FROM THE RESPONSE TO THE SAME DOSE OF SYNTOCINON

The numerals in brackets give the number of pairs of observations on which the comparison was made.

	Blood Flow		Blood Pressure	Pulse Rate
	Hand	Forearm		
Intravenous injection	+68 (16)	+65 (14)	+12 (13)	+86 (13)
Continuous infusion	+120 (4)	+57 (4)	+23 (4)	+38 (4)
Intra-arterial injection	+38 (11)	−23 (4)	0	0

DISCUSSION

The synthetic oxytocin, Syntocinon, has been found to have the same activity as natural oxytocin when tested by a variety of methods of assay, including the isolated rat uterus, the chicken blood pressure, milk ejection pressure in rabbits, cat uterus *in situ*, blood pressure and antidiuretic effects (Berde, Doeppner, and Konzett, 1957). Synthetic analogues of oxytocin, phenylalanyl[3]-oxytocin, leucyl[3]-oxytocin, glutaminyl[3]-oxytocin, and valyl[3]-oxytocin differ, however, in some respects in their relative potencies (Berde *et al.*, 1957). In particular, it has been shown that valyl[3]-oxytocin, standardized by the rat uterus and chicken blood pressure methods, has about four times the oxytocic effect of Syntocinon on the human uterus (Smyth, 1958).

The present experiments indicate that valyl[3]-oxytocin, again standardized using the rat uterus and chicken blood pressure, also has a greater effect than Syntocinon on the cardiovascular system in man, being about 1.5 to 2 times as active a vasodilator.

These observations, apart from a possible therapeutic bearing, illustrate the fact that specific actions of these cyclic octapeptides are independent of each other and depend on particular amino-acid groupings within the molecule.

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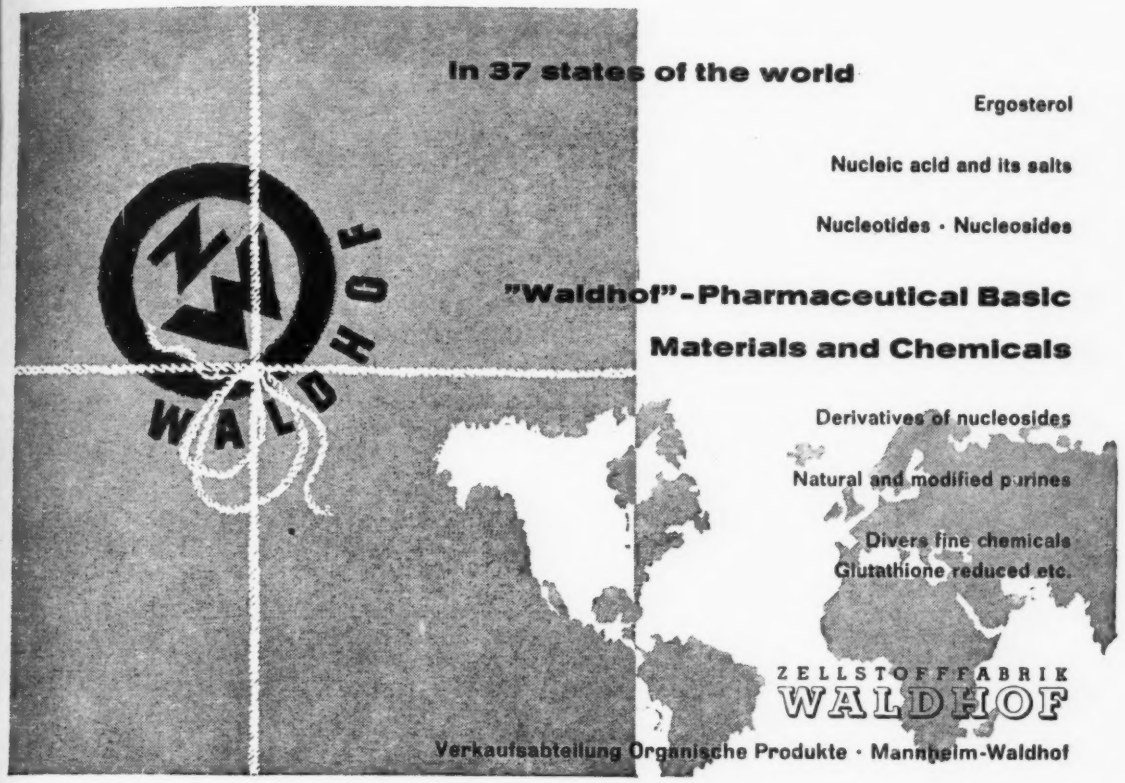

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